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1: FEMS Microbiol Rev. 1999 Jun;23(3):277-95.

3'-End processing of pre-mRNA in eukaryotes.

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3'-Ends of almost all eukaryotic mRNAs are generated by endonucleolytic cleavage and addition of a poly(A) tail. In mammalian cells, the reaction depends on the sequence AAUAAA upstream of the cleavage site, a degenerate GU-rich sequence element downstream of the cleavage site and stimulatory sequences upstream of AAUAAA. Six factors have been identified that carry out the two reactions. With a single exception, they have been purified to homogeneity and cDNAs for 11 subunits have been cloned. Some of the cooperative RNA-protein and protein-protein interactions within the processing complex have been analyzed, but many details, including the identity of the endonuclease, remain unknown. Several examples of regulated polyadenylation are being analyzed at the molecular level. In the yeast Saccharomyces cerevisiae, sequences directing cleavage and polyadenylation are more degenerate than in metazoans, and a downstream element has not been identified. The list of processing factors may be complete now with approximately a dozen polypeptides, but their functions in the reaction are largely unknown. 3'-Processing is known to be coupled to transcription. This connection is thought to involve interactions of processing factors with the mRNA cap as well as with RNA polymerase II.

Publication Types:
- Review
- Review, Tutorial

PMID: 10371034 [PubMed - indexed for MEDLINE]

1: Microbiol Mol Biol Rev. 1998 Jun;62(2):465-503.

Molecular genetics of the RNA polymerase II general transcriptional machinery.

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Transcription initiation by RNA polymerase II (RNA pol II) requires interaction between cis-acting promoter elements and trans-acting factors. The eukaryotic promoter consists of core elements, which include the TATA box and other DNA sequences that define transcription start sites, and regulatory elements, which either enhance or repress transcription in a gene-specific manner. The core promoter is the site for assembly of the transcription preinitiation complex, which includes RNA pol II and the general transcription fctors TBP, TFIIB, TFIIE, TFIIF, and TFIIH. Regulatory elements bind gene-specific factors, which affect the rate of transcription by interacting, either directly or indirectly, with components of the general transcriptional machinery. A third class of transcription factors, termed coactivators, is not required for basal transcription in vitro but often mediates activation by a broad spectrum of activators. Accordingly, coactivators are neither gene-specific nor general transcription factors, although gene-specific coactivators have been described in metazoan systems. Transcriptional repressors include both gene-specific and general factors. Similar to coactivators, general transcriptional repressors affect the expression of a broad spectrum of genes yet do not repress all genes. General repressors either act through the core transcriptional machinery or are histone related and presumably affect chromatin function. This review focuses on the global effectors of RNA polymerase II transcription in yeast, including the general transcription factors, the coactivators, and the general repressors. Emphasis is placed on the role that yeast genetics has played in identifying these factors and their associated functions.

Publication Types:
- Review
- Review, Tutorial

1: Acta Biochim Pol. 1998;45(4):869-81.

Recognition of the 5' splice site by the spliceosome.

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The splicing of nuclear pre-mRNAs is catalyzed by a large, multicomponent ribonucleoprotein complex termed the spliceosome. Elucidation of the molecular mechanism of splicing identified small nuclear RNAs (snRNAs) a important components of the spliceosome, which, by analogy to the self-splicing group II introns, are implicated in formation of the catalytic center. In particular, the 5' splice site (5'SS) and the branch site, which represent the two substrates for the first step of splicing, are first recognized by U1 and U2 snRNPs, respectively. This initial recognition of splice sites is responsible fo the global definition of exons and introns, and represents the primary target for regulation of splicing. Subsequently, pairing interaction between the 5'SS and U1 snRNA is disrupted and replaced by a new interaction of the 5'SS with U6 snRNA. The 5'SS signal contains an invariant GU dinucleotide present at the 5' end of nearly all known introns, however, the mechanism by which the spliceosome recognizes this element is not known. We have identified and characterized a specific UV light-induced crosslink formed between the 5'SS RNA and hPrp8, a protein component of U5 snRNP in the spliceosome that is likely to reflect a specific recognition of the GU dinucleotide for splicing. Because recognition of the 5'SS must be linked to formation of the catalytic site, the identification of a specific and direct interaction between the 5'SS and Prp8 has significant implications for the rol of this protein in the mechanism of mRNA splicing.

Publication Types:
- Review
- Review, Tutorial

PMID: 10397335 [PubMed - indexed for MEDLINE]

Protein Engineering vol.4 no.6 pp.691–693, 1991

Isolation and characterization of mutants of firefly luciferase which produce different colors of light

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The luciferase cDNA from the 'Genji' firefly, *Luciola cruciata*, was mutated with hydroxylamine to isolate mutant luciferases. Some of the isolated mutant enzymes produced different colors of light, ranging from green to red. Five such mutants, producing green (λ_{max} = 558 nm), yellow-orange (λ_{max} = 595 nm), orange (λ_{max} = 607 nm) and red light (λ_{max} = 609 and 612 nm), were analyzed. The mutations were found to be single amino acid changes, from Val239 to Ile, Pro452 to Ser, Ser286 to Asn, Gly326 to Ser and His433 to Tyr respectively.

Key words: color mutant/firefly luciferase/random mutagenesis/wavelength of maximum intensity

Introduction

Firefly luciferase catalyzes the production of light from luciferin in the presence of ATP, Mg^{2+} and molecular oxygen (Deluca and McElroy, 1978). This enzyme efficiently converts chemical energy into light with a quantum yield of 0.88 (Seliger and McElroy, 1960). Due to its high sensitivity and extreme specificity for ATP, luciferase has been used for assay of ATP in various biological samples (Ludin, 1981).

The luciferase cDNA from the Japanese firefly, *Luciola cruciata* ('Genji-botaru' in Japanese), has been cloned and analyzed in our laboratory (Masuda *et al.*, 1989). The primary structure of this luciferase deduced from the nucleotide sequence was shown to consist of 548 amino acids, with a total molecular weight of 60 024. This luciferase catalyzes a reaction that produces yellow-green light (λ_{max} = 562 nm), which is the same as that emitted by the North American firefly (Seliger and McElroy, 1964). It has been shown that the colors of light emitted by fireflies vary among species from green to yellow (λ_{max} = 543–582 nm) (Seliger and McElroy, 1964). Since the substrate (D-luciferin) is the same for all species, the differences in the color of the light must be due to variations in the structure of the enzymes (McElroy and Seliger, 1966). Recently, cDNAs of luciferase from the bioluminescent click beetle, *Pyrophorus plagiophthalamus*, were cloned and their nucleotide sequences determined (Wood *et al.*, 1989a). These cDNA clones code for luciferases of four different types, distinguished by the colors of their bioluminescence. The amino acid sequences of these luciferases are 95–99% identical, and less than two or three amino acid changes are needed for the spectral shift in the color (Wood *et al.*, 1989b,c).

In the course of mutagenesis studies of luciferase cDNA from *Luciola cruciata*, we found that some mutants emitted different colors of light. Sequence analysis of these mutants revealed that the mutations were single amino acid changes.

Materials and methods

Plasmid, Escherichia coli strain and media

Plasmid pGLf37 was constructed from pGLf1 by Mr H.Tatsumi in our laboratory (Masuda *et al.*, 1989). *Escherichia coli* strain JM 101 (*SupE, thi,* Δ(*lac-pro*), [F'*traD*36, *lacI Z* ΔM15, *proAB*]) (Yanish-Perron *et al.*, 1985) was used for the expression of luciferase cDNA. The *E.coli* cells were grown in LB broth (1% Difco tryptone/0.5% yeast extract/0.5% sodium chloride), and 50 μg/ml ampicillin was added when necessary.

Mutagenesis and screening of 'color' mutants

Plasmid pGLf37 containing Genji-firefly luciferase cDNA was treated, according to the methods of Kironde *et al.* (1989), with 0.8 M hydroxylamine/0.1 M sodium phosphate/1 mM ethylenediaminetetraacetic acid (EDTA), pH 6.0, for 2 h at 65°C (Figure 1). The mutagen-treated plasmid was precipitated with ethanol and redissolved in 10 mM Tris–HCl/1 mM EDTA, pH 8.0, followed by transformation into *E.coli* JM 101. After 12 h at 37°C, colonies on LB/ampicillin plates were transferred to nitrocellulose filters. The filters were soaked with 0.5 mM luciferin in 100 mM sodium citrate buffer, pH 5.0 (Wood and DeLuca, 1987), and the colors of bioluminescence emitted by the colonies were monitored.

Purification of luciferase

Escherichia coli JM 101 cells harboring the mutant plasmid were cultured in 3 ml of LB broth containing ampicillin at 37°C for 12 h. The cultures, 2 ml each, were inoculated into 100 ml of LB broth containing ampicillin. After growth at 37°C for 6 h, the cultures were harvested. *Escherichia coli* pellets were resuspended in lysis buffer (100 mM potassium phosphate, pH 7.8/2 mM EDTA/1 mg lysozyme per ml), incubated on ice for 15 min and then frozen on dry ice. The frozen pellets were allowed to thaw at 25°C and cleared by centrifugation.

The lysates of *E.coli* were fractionated with ammonium sulfate; the fraction precipitated between 0.3 and 0.6 saturation was saved. The precipitate was dissolved with 25 mM Tris–HCl buffer, pH 7.8/1 mM EDTA/10%-saturated ammonium sulfate, and then loaded on an Ultrogel AcA34 gel filtration column (LKB). The active fraction was applied to a hydroxyapatite column (Tosoh, Tokyo, Japan), followed by elution with a 10–100 mM sodium phosphate gradient.

DNA sequencing

Various restriction fragments derived from mutant luciferase cDNA were subcloned into pUC118 or pUC119, and sequenced using a DNA sequencer model 373A (Applied Biosystems).

Results and discussion

Screening of 'color' mutants

As shown in Figure 1, pGLf37, which is a plasmid directing the synthesis of active luciferase in *E.coli* under control of the *trp* promoter, was treated with hydroxylamine solution for 2 h at

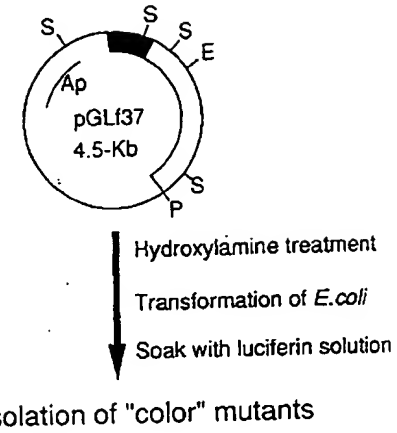

Fig. 1. Strategy of mutation. Plasmid pGLf37 containing Genji firefly luciferase cDNA was treated according to Materials and methods. Clear and solid portions show the luciferase cDNA and *trp* promoter respectively. Abbreviations: E, *Eco*RV; P, *Pst*I; S, *Ssp*I; Ap, ampicillin resistance gene.

Fig. 2. The color of bioluminescence emitted by the wild-type and mutant luciferases. Left to right: yellow-green (wild-type luciferase); yellow-orange; orange; red; green; yellow. These luciferases were purified according to Materials and methods. Ten microliters of these luciferases were added to 400 μl of substrate mix (25 mM glycylglycine, pH 7.8/5.4 mM $MgSO_4$/0.086 mM luciferin/2 mM ATP) to confirm the bioluminescence.

65°C. The plasmid was transformed into *E.coli* JM 101. To monitor the colors of bioluminescence, the transformants were soaked with luciferin solution. Subsequently, we isolated several mutants, producing colors of light varying from green to red.

Bioluminescence spectra of mutant luciferases

Mutant luciferases were purified to homogeneity as described in Materials and methods. Figure 2 shows the colors of light emitted by the purified enzymes. When their spectra were measured with a IMUC-7000 intensified multichannel photodetector (Otsuka Denshi, Osaka, Japan), the wavelengths of maximum intensity were 558 nm for green, 565 nm for yellow, 595 nm for yellow-orange, 607 nm for orange and 609 and 612 nm for red (Table I).

It is known that luciferase from the American firefly produces light with a peak intensity at ~560 nm (yellow-green) under optimal conditions (Seliger and McElroy, 1964). However, this peak can be affected by temperature, pH and metal ions. At low pH or in the presence of heavy metals, the emission peak is shifted toward the red, showing an emission of ~ 615 nm, but with a marked decrease in the quantum yield of the reaction (Seliger and McElroy, 1960, 1964). This phenomenon is also observed for Genji firefly luciferase. In the mutants C-M-1, 2, 3, 4 and 11, by contrast, the spectral peaks were shifted toward longer wavelengths under optimal conditions, with no detectable decrease of light intensity. Moreover, for mutants C-M-3 (red)

Table I. Wavelength of maximum intensity of light from wild-type and mutant luciferases

Luciferase	Color[a]	λ_{max} (nm)	
		pH 7.8	pH 6.0
Genji	yellow-green	562	609
C-M-1	orange	607	614
C-M-2	red	609	611
C-M-3	red	612	612
C-M-4	yellow-orange	595	609
C-M-6	green	558	558
C-M-11	yellow	565	612

The spectra were measured with a IMUC-7000 intensified multichannel photodetector at pH 7.8 and pH 6.0. Except for pH, the condition for luminescence was the same as described in Figure 2.
[a]Color was confirmed at pH 7.8.

Table II. DNA sequence and amino acid sequence change in mutants

Mutant	Color	Base change	Amino acid change
C-M-1	orange	G857 → A	Ser286 → Asn
C-M-2	red	G976 → A	Gly326 → Ser
C-M-3	red	C1297 → T	His433 → Tyr
C-M-4	yellow-orange	C1354 → T	Pro452 → Ser
C-M-6	green	G715 → A	Val239 → Ile

and C-M-6 (green) there was no pH effect on emission spectra (Table I). For the other red (C-M-2), orange (C-M-1) and yellow-orange (C-M-4) mutants, the shift of spectral peak at pH 6.0 was detected slightly, but was not so large as for Genji luciferase. On the other hand, for the yellow mutant C-M-11, the shift was largest in the mutant luciferases and its reaction decay was similar to that for Genji luciferase. In contrast, the reaction decay in the C-M-3 and C-M-6 mutants at pH 6.0 was not as great as that of Genji wild-type luciferase (data not shown). It is uncertain whether the color shifts and decay rates of the light output at low pH are related to each other.

Amino acid sequences of mutants

Determination of the nucleotide sequences in some mutant luciferases (green, yellow-orange, orange and red) was carried out to identify the base changes present. As shown in Table II, the change in the yellow-orange mutant was found to be from CCA to TCA, resulting in a change of Pro to Ser at position 452. In the orange mutant, the alteration was from Ser to Asn at position 286. In the two red mutants, the changes for C-M-2 and C-M-3 were identified as Gly326 to Ser and His433 to Tyr respectively. The green mutant contained a change of Val to Ile at position 239. These results indicate that only a single amino acid substitution in a luciferase molecule is enough to produce the change in bioluminescence color.

Recently the nucleotide sequences of click beetle luciferase cDNAs were determined by Wood *et al.* (1989a). These are of four different types, distinguishable by the colors of light produced by the luciferases they code: green, yellow-green, yellow and orange. Fragments of the four different types were recombined to construct hybrid luciferases, and two groups of amino acids, each capable of producing a change in the spectrum of luciferase greater than 16 nm, were detected (Wood *et al.*, 1989b). For the first set, which contains the changes Arg223 to Glu and Leu238 to Val, the spectrum shifts from 560 to 577 nm. The spectrum for the other set, containing the changes Ser247 to Gly, Asp352 to Val and Ser358 to Thr, shifts from 560 to

580 nm. It is not known whether all the amino acids in each set are required for the spectral change.

In our experiments the colors of light were shown to be changed effectively by only one amino acid substitution (Table II). Four mutants showed an upward spectral shift of >30 nm, and the wavelength of their maximum light intensity was far longer than the orange of click beetle luciferase (593 nm). A red mutant (C-M-3), containing a change of His433 to Tyr, showed an especially large upward shift of 50 nm to 612 nm.

When the sequences of the mutant luciferases were compared with those of click beetle enzymes, no common amino acid sequence affecting the color of light was detected. Further, in the color mutants of firefly luciferase, there was no reversal of hydrophobicity or a large change in the conformational parameters of the secondary structure. For example, in the green mutant, the amino acid alteration was from Val to Ile. Both of these are hydrophobic, and conformational parameters for the α-helix and β-sheet together with their charges are also similar (Chou and Fasman, 1978). When we estimated the secondary structures of wild-type and mutant luciferases from each of the primary structures using the algorithm of Garnier *et al.* (1978), no drastic structural changes were detected. Thus, it may be concluded that the different colors of bioluminescence were caused by only subtle differences in the tertiary structure of the luciferase molecule. It would be of interest to discover whether the color change, which is a unique parameter for luciferase, is related to the other enzymatic properties. Further studies, including examination of the properties of these color mutant enzymes and analysis of their tertiary structures, would elucidate the relationship between the structure and function of luciferase.

Firefly luciferase has been used for assay of ATP in various biological samples (Ludin, 1981). The mutant luciferases described above could be used more effectively for determining the amount of ATP in colored samples. In the case of red-colored samples, determination was found to be twice as sensitive using the mutant enzyme producing red light than with wild-type luciferase (data not shown).

Hydroxylamine treatment used in this study was a very simple and efficient method for introducing random base substitutions into luciferase cDNA. However, since this chemical mutagen causes only GC to AT transition mutations, mutants with limited substitution of amino acids can be obtained. To overcome this problem, Myers *et al.* (1985) used the method of treating single-stranded DNA with nitrous acid, formic acid and hydrazine, followed by the synthesis of the complementary strand with reverse transcriptase. Using this method for luciferase, various color mutants which cannot be found at present may be obtained.

In the present study, we succeeded for the first time in isolating several 'color' mutant luciferases. Sequence analysis revealed that only a single amino acid change in the 548 amino acids of luciferase is enough for the color variation. Analysis of other mutant luciferases is now in progress.

Acknowledgements

We wish to thank Mr Hiroki Tatsumi for pGLf37 and Mrs Kazuko Saitoh for her technical assistance. We also wish to thank Mrs Hideko Otake for critical reading of the manuscript.

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JOURNAL OF BIOLUMINESCENCE AND CHEMILUMINESCENCE VOL 4 289–301 (1989)

Introduction to Beetle Luciferases and their Applications

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All beetle luciferases have evolved from a common ancestor: they all use ATP, O_2, and a common luciferin as substrates. The most studied of these luciferases is that derived from the firefly *Photinus pyralis*, a beetle in the superfamily of Cantharoidea. The sensitivity with which the activity of this enzyme can be assayed has made it useful in the measurement of minute concentrations of ATP. With the cloning of the cDNA coding this luciferase, it has also found wide application in molecular biology as a reporter gene. We have recently cloned other cDNAs that code for luciferases from the bioluminescent click beetle, *Pyrophorus plagiophthalamus*, in the superfamily Elateroidea. These newly acquired luciferases are of at least four different types, distinguishable by their ability to emit different colours of bioluminescence ranging from green to orange. Unique properties of these luciferases, especially their emission of multiple colours, may make them additionally useful in applications.

Keywords: Firefly luciferase; click beetle luciferases; reporter genes

INTRODUCTION

Man's perception of the world is visually oriented. Since bioluminescence is one of the few things that can be seen in the dark, it is understandable that this has been a topic of biochemical research for many decades. Fireflies have been prominent in this research endeavour, in part because they are abundant and their light organs are replete with luciferase. Thus they provided a plentiful resource for further study. Early research on fireflies was done primarily to better understand this peculiar phenomena of living light. Sometimes, though, the earliest work was justified as a means of developing artificial lighting. In the late 1940s, when the general importance of ATP metabolism was just becoming recognized, it was discovered that ATP was a component in the luminescent reaction of fireflies. The firefly luciferase became one of the paradigms of ATP-utilizing enzymes. Because of the extreme sensitivity with which the activity of this enzyme could be assayed, it was soon adapted as a tool in the measurement of very low concentrations of ATP. Subsequently, luciferase was combined with other ATP-utilizing enzymes to produce coupled enzymatic systems. In these systems, the luciferase was the reporter allowing sensitive measurements of a wide variety of metabolites.

Recently, firefly luciferase has found new application as a reporter of genetic activity in

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living cells. Its application in this area was made possible by the cloning of its cDNA, which can direct the synthesis of active enzyme in foreign hosts (de Wet *et al.*, 1985). In this report, we describe briefly how the properties of luciferase have made it well suited for this purpose. We also present information on the recent cloning of several new cDNAs from a bioluminescent click beetle (Wood *et al.*, 1988a). These cDNA clones encode for four different types of luciferases, which can be distinguished by their ability to emit different colours of bioluminescence. These colours range from green to orange. Structurally, the click beetle luciferases differ significantly from the firefly luciferase, and these differences are reflected in their chemical properties. Because of this, the click beetle luciferases may have additional features to make them useful as genetic reporter.

PROPERTIES OF THE FIREFLY LUCIFERASE

All beetle luciferases catalyse the conversion of chemical energy into light by a two-step process (Fig. 1)(Seliger and McElroy, 1964; DeLuca and McElroy, 1978). This process utilizes ATP, O_2, and beetle luciferin, a unique heterocyclic acid found only in bioluminescent beetles. In the first step, the carboxylate group of luciferin is activated by acylation with the alpha-phosphate of ATP. The luciferyl adenylate is then oxidized with molecular oxygen, in the second step, to yield AMP, carbon dioxide, and oxyluciferin. The oxyluciferin is generated in an electronically excited state which, upon transition to the ground state, emits the photon characteristic of bioluminescence. For firefly luciferase, the most studied of the beetle luciferases, the quantum yield for this reaction has been measured at 0.88 relative to the consumption of luciferin (McElroy and Seliger, 1960). This is the highest yield reported for any luminescent reaction.

Under optimal conditions the firefly luciferase emits light whose peak intensity is at 561 nm (yellow-green). This is the same as the light emitted from live fireflies. Under a variety of conditions, however, the structure of luciferase can be altered to a form which emits predominately at 617 nm (red) (Seliger and McElroy, 1964). Some conditions which can cause this spectral shift are pH below 7.5, temperature above 20 °C, the presence of denaturants such as urea, and the presence of heavy metals such as Zn^{2+}, Cd^{2+}, or Hg^{2+} (Seliger and McElroy, 1964). Some chemical modifications of the enzyme, or the use of substrate analogues, can also cause the enzyme to emit red light (DeLuca *et al.*, 1973; Alter and Deluca, 1986). In the case of pH, the shift to red light is associated with a substantial decrease in the quantum yield of the reaction (McElroy and Seliger, 1966). This decrease in quantum yield is probably evident under any condition that promotes the red-emitting form. The spectral shift associated with changes in temperature, or the presence of denaturants, can be interpreted as resulting from partial unfolding of the enzyme structure. For others conditions, it is not known whether the effects are localized to key reactive residues, or whether they also cause general perturbations to the structure. Aside from the actual decrease in the quantum efficiency of luciferase in the red-emitting form, the spectral shift also causes an apparent decrease in enzymatic activity. This is because photomultiplier tubes are generally much less sensitive to red light than green light. Both these real and apparent affects combine to give a large pH dependence to the measured light output of firefly luciferase. The optimal pH for light output is pH 7.8.

Under conditions of excess substrates, the light output of luciferase is proportional to the

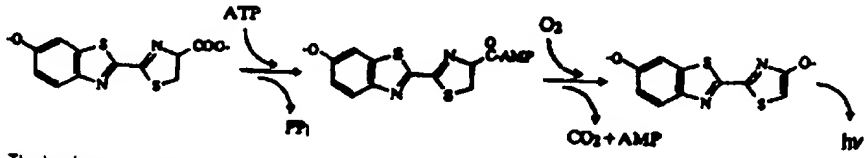

Figure 1. The luminescent reaction catalysed by beetle luciferase. Beetle luciferin is the heterocyclic molecule depicted on the left. Other reactants are depicted by their common abbreviations. The final product, *hν*, is the photon of light emitted from oxyluciferin during its transition to its ground-state electronic configuration

concentration of enzyme over at least a 10,000-fold range. With the use of a sensitive luminometer, as little as 10 femtograms of enzyme can be detected (10^5 molecules). Initiation of the luminescent reaction by rapid mixing of the substrates with the enzyme results in a rapid release of light which reaches maximum intensity after about 0.3 seconds. The intensity is then quickly inhibited in a biphasic manner, reaching about 10% of its peak value after 30 seconds. Beyond one minute the intensity is produced from a steady-state process which decays slowly over several hours. Because the peak luminescence is typically over 10-fold greater than the steady-state luminescence, the enzymatic assay is most sensitive when the first 30 seconds of the reaction are included in the measurement of total light output. This is typically accomplished in a luminometer where the reaction is initiated in front of the photomultiplier tube by injection of substrates directly into the sample holder.

Firefly luciferase is a 61 kD enzyme which apparently is active as a monomer. It is coded by a 550 amino acid reading frame in its mRNA, and is probably produced as an active enzyme without the necessity of post-translational modifications. In the firefly, the enzyme is located in specialized peroxisomes of the light organ (Keller *et al.*, 1987). It is directed to these sub-cellular organelles by a targeting sequence at the C-terminus of the protein (Gould and Subramani, 1988b, 1988c). This targeting sequence is conserved throughout eukaryotes, and will cause the luciferase to localize in the peroxisomes of other organisms when expressed in exogenous hosts.

APPLICATION OF FIREFLY LUCIFERASE AS A REPORTER GENE

With the past decade have come dramatic advancements in our ability to manipulate genetic materials. Enabled by these new techniques, the study of sub-cellular events which regulate genetic activity has become one of the largest areas of research today. A key tool in this area of research is the reporter gene, which provides an observable parameter in the monitoring of genetic events at a molecular level. In its simplest form, a reporter gene is a fragment of DNA which encodes for an easily detectable protein. This protein is the reporter. In experiments, the reporter gene is linked to other fragments of DNA which are thought to contain genetic control elements, and the assemblage is introduced into living cells. Production of the reporter in the cell is regulated by the action of the control elements on the transcriptional activity of the reporter gene. Thus, the reporter is the observable parameter allowing the experimenter to monitor the action of the control elements.

In practice, the transcriptional activity of a reporter gene can be quite low, and experiments are often limited by an inability to detect the reporter. Therefore, for a reporter to be widely useful, it must be detectable in very low concentrations. In addition, the reporter must be detectable by a method that can distinguish it from other proteins native to the host cell. Firefly luciferase meets these criteria ideally. It can be detected in very small amounts through its bioluminescent activity, and since bioluminescence is not a common event in living systems, its activity will be unique in the experimental host. That is, there is no endogenous luminescent activity of the host to interfere with the detection of even miniscule amounts of luciferase. The bacterial enzyme, chloramphenicol acetyltransferase (CAT), has been used conventionally as a reporter in eukaryotic systems for similar reasons. Its enzymatic activity is not found in eukaryotic cells, so CAT can also be detected without confusion from host actitivities. Its assay is based on conversion of the antibiotic chloramphenicol to mono- and di-acetylated forms. High sensitivity is provided by the use of ^{14}C-labelled chloramphenicol as the substrate. This method requires that the products of the reaction be separated from the substrate before quantification, usually by thin layer chromatography or HPLC.

Because CAT is widely used as a genetic reporter, it was used as a benchmark to evaluate the suitability of firefly luciferase in this application (de Wet *et al.*, 1987). It was found that the levels of expression of CAT and luciferase in eukaryotic systems were comparable. It had been previously shown that the production of CAT in eukaryotic cells is proportional to mRNA transcription from the reporter gene. Since luciferase production paralleled CAT production under a variety of experimental conditions, luciferase must also be a proportional indicator of transcriptional activity. However, because of the efficient detection methods achievable with bioluminescence, the assay of luciferase is 100 to 1000 times

more sensitive. Thus, much lower levels of genetic activity are detectable. Furthermore, the time required to assay luciferase is much less than CAT. Using a luminometer or scintillation counter, the luciferase assay requires about a minute per sample. The CAT assay usually requires several hours. The assay of luciferase also does not require the special precautions needed for radioactive ^{14}C.

One of the unique advantages of firefly luciferase as a reporter of genetic activity is its potential to measure this activity from within living cells. This is not possible with use of CAT since the products of the reaction require separation from the assay mixture in order to be quantified. The photons produced in the luciferase reaction, however, are generally able to pass from within the host cell to allow external detection. A precondition of this is that the luciferin substrate be able to pass into the cell to combine with the luciferase reporter. The other substrates of the reaction, ATP and O_2, are readily available in the interior of the cell. The mere addition of luciferin to the external media is sufficient to allow its passage across the cellular membrane. But the light output elicited by this method is less than expected given the extent of luciferase contained within the cells. Light output can be increased with the use of permiablizing agents such as DMSO or nigericin (Gould and Subramani, 1988a), but still not to the full potential expected. It is not known whether permeability of the outer membrane is the only limitation, or whether there are other inhibitors of activity. One possibility is that the peroxisomal membrane acts as a second barrier to luciferin passage. Since luciferase is localized into peroxisomes, most of the luminescent activity may arise from these organelles. Experiments are currently under way to remove the peroxisomal targeting signal from luciferase so that it will remain in the cytoplasm. This may improve its ability to elicit luminescence from within intact cells. However, other possibilities exist, such as unfavourable microenvironmental effects, which could inhibit the activity of luciferase in a foreign host.

Since the first published reports of its use as a genetic reporter, this new application of firefly luciferase has received much interest. By the time this article was written, we had received approximately 1000 requests for the cDNA encoding luciferase from other laboratories wishing to apply it to their experimental systems. The feedback from these other laboratories has been quite positive. In most cases, researchers are finding that the use of luciferase instead of CAT is saving much time in the execution of their experiments. The time saved is not only in the much shorter assay time of luciferase, but also in the time required for sufficient expression of the reporter. Previously, production of the reporter often was not detectable for 24 to 48 hours after the reporter gene was introduced into cells. Because of the much higher sensitivity of the luciferase assay, expression of the reporter gene can typically be measured after only a few hours. In some cases, where expression of the reporter was previously too low for detection under any conditions, the use of luciferase has allowed measurements to be made. To date, luciferase has been expressed from its cDNA in almost every living kingdom. It has been expressed in bacteria (de Wet *et al.*, 1985), yeast (Wood and DeLuca, 1987), dictyostelium (Howard *et al.*, 1988), mammalian cells (de Wet et al., 1987, Gould and Subramani, 1988a), and plant cells (Ow *et al.*, 1986), as well as in transgenic mice (DiLella *et al.*, 1988; Crenshaw and Rosenfeld, 1988) and plants (Ow *et al.*, 1986).

COMPARISON OF FIREFLY AND CLICK BEETLE LUCIFERASES

Bioluminescent beetles are found in two superfamilies, Elateroidea and Cantharoidea (Lloyd, 1978). Fireflies are members of the family Lampyridae in the superfamily Cantharoidea; as indicated above, they have been the primary source of a beetle luciferase because of their abundance and accessibility. In the superfamily Elateroidea, only the family Elateridae contains bioluminescent members, which are more commonly known as click beetles. This family of beetles is one of the most widely distributed, with species found in most areas of the world. However, unlike Lampyridae, where nearly all of the members are bioluminescent, only a small percentage of Elateridae are so. Most of these are located in the Caribbean and in South America. Their taxonomy suggests that the click beetles are the most evolutionarily distant of the bioluminescent beetles from the fireflies (Crowson, 1981). The time of divergence of the Elateroidea and Cantharoidea superfamilies cannot be estimated directly owing to a lack of fossils. But by

comparison of the morphological differences between these groups of beetles, corroborated with the fossil record of other beetles, it has been estimated that these superfamilies diverged about 120 million years ago.

Morphologically the click beetles and fireflies are quite distinct (Fig. 2). The click beetles have a hard exoskeleton, and are often larger than the fireflies. They can be recognized by a characteristic behaviour they display when being constrained or placed on their backs. They make an audible 'click' sound by forcibly arching their head forward. Bioluminescent click beetles have two sets of light organs. One pair is located on the dorsal surface of the head. These light organs emit long pulses of light when the beetles are not in flight. The second set is a single organ located in a cleft on the ventral surface of the beetle between the mesothorax and abdomen. This light organ also emits long pulses of light but only when the beetle is in flight. On the ground the cleft is closed and the light is extinguished. For most species of bioluminescent click beetle, the ventral organ emits light at a longer wavelength than the dorsal organ. The position and activity of the light organs in fireflies is quite different. These beetles have one set of light organs located on the ventral surface of the abdomen, on the posterior sternites. They generally emit short burst of light in a pattern which is indicative of the particular species.

One species of click beetle has been of particular interest since its bioluminescence was first studied in 1963. This species, *Pyrophorus plagiophthalamus*, is an especially large click-beetle being typically 3 cm in length. It was of interest because its bioluminescence presents an unusually large range of colorus (Seliger *et al.*, 1964). Furthermore, the colours vary between individuals, a property not found in fireflies. The light of the dorsal organ is greenish in colour, but varies between individuals from green (548 nm) to yellow-green (565 nm). The ventral organ varies over a much wider range, from green (547 nm) to orange (594 nm) (Biggley *et al.*, 1967). The luciferases of these beetles were extracted to determine the source of these different colours. In extracts, the bioluminescence spectra were not different from those of the living beetles. Analysis showed that the different colours were not due to alterations of the substrates of the reaction, which are the same as utilized by the firefly luciferase. It was concluded that the differences were due to variation in the interaction of the substrates with the luciferases (Seliger and McElroy, 1964). Unfortunately, attempts to study these luciferases further were limited by the difficulty of collecting sufficient quantities of the beetles.

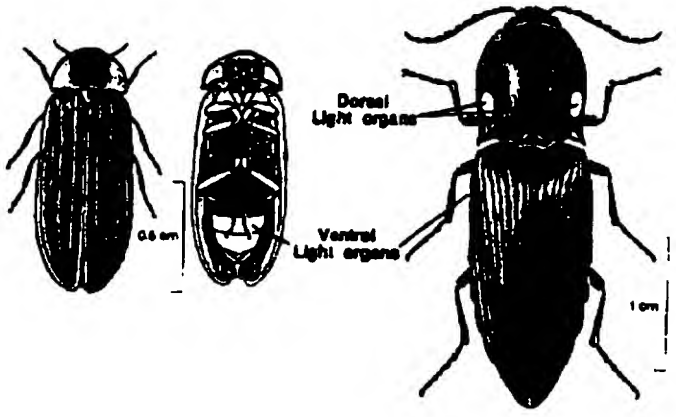

Figure 2. The general morphology of fireflies and click beetles. A firefly (Lampyridae) is shown in two views on the left, and a click beetle (Elateridae) is shown on the right.

CLONING AND EXPRESSION OF CDNAS ENCODING CLICK BEETLE LUCIFERASES

Expression of firefly luciferase in *Escherichia coli* demonstrated that we could produce this enzyme from a source which was easily grown in the laboratory. Thus, in this case, the information contained in the cDNA encoding firefly luciferase was in itself sufficient to generate an active enzyme in a foreign host. Application of this technology to the click beetle luciferases could circumvent the problems of collecting large quantities of the beetles. The methods used in cloning a cDNA which encodes luciferase require only a small supply of the beetles, and they are needed only once. Afterwards, bacterial hosts generate the DNA and enzyme needed for further study. Production of the click beetle luciferases from cDNA clones has the additional advantage that genetic variants of the enzyme, such as those which produce the different colours of bioluminescence, are generated in isolation of one another. Enzymes isolated from the click beetles directly would require methods for separation of the different variants. This would be difficult since, as was subsequently found, the physical differences between these variants are few. Furthermore, the amino acid sequences of the luciferases can be determined from the DNA sequences of their cDNA clones. DNA sequencing is an efficient technique making it practicable to determine the amino acid sequence differences between several proteins of over 500 amino acids each.

Specimens of *Pyrophorus plagiophthalamus*, collected from the northeast end of Jamaica, were transported live to the laboratory and frozen in liquid N_2. Messenger RNA was isolated from ventral light organs of approximately 60 beetles, one microgram of which was converted to cDNA (de Wet *et al.*, 1986). This was packaged in a specialized lambda cloning vector, Lambda ZAP, to yield 700,000 recombinant plaques (Fig. 3) (Short *et al.*, 1988). We had originally intended to screen the library by DNA hybridization using the cDNA sequence of firefly luciferase. However, attempts at visualizing the click beetle luciferase gene in Southern blots, using the firefly luciferase cDNA as the probe, failed to demonstrate cross-hybridization even under conditions of low stringency. It had been previously shown that antibodies raised against firefly luciferase can cross-react with the click beetle luciferases

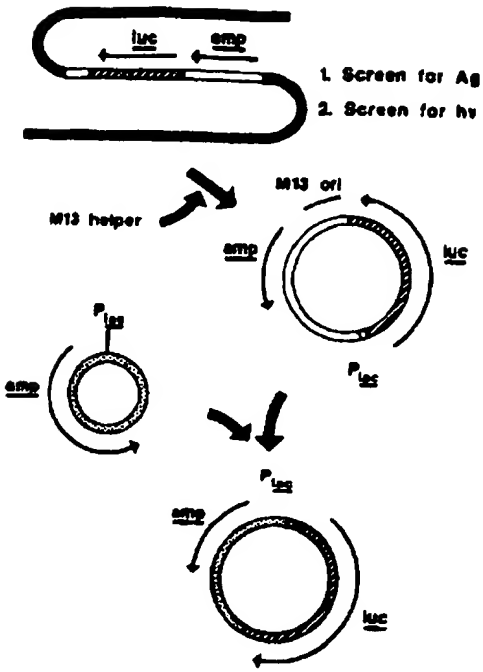

Figure 3. The strategy for cloning and expressing the cDNAs coding click beetle luciferases. The upper left corner depicts the Lambda ZAP vector. The cDNA library in this form was screened for the expression of antigenic polypeptides (Ag). With the use of M13 helper, Lambda ZAP was transformed either individually or *en bloc* into Bluescript plasmids (upper right). The cDNA library in this form was screened for the expression of luminescence (*hv*). The expression of luminescence was improved by transferring the cDNA clones to a vector containing the *tac* promoter (lower left and bottom)

(Wienhausen and DeLuca, 1985). Thus we used such antibodies, raised in rabbits, to screen the cDNA library. Because the cross-reactivity with click beetle luciferases is weak, we used antibodies that had been affinity purified by selection on a Sepharose column containing immobilized firefly luciferase (a gift from Dr Gilbert Keller; Keller *et al.*, 1987).

The original screening was done on unamplified aliquots of the lambda cDNA library. Determined by the colorimetric detection of alkaline phosphatase conjugated to anti-rabbit antibodies, 5.5% of the recombinant lambda phage expressed luciferase antigens. Eighteen clones were chosen for further analysis. A unique

feature of the Lambda ZAP cloning vector is that it can be transformed into a bacterial expression plasmid (Bluescript) by an *in vivo* process involving the addition of an M13 helper phage (Short *et al.*, 1988). The recombinant lambda containing the four longest cDNA clones were transformed into their plasmid form, and found to be able to express bioluminescence in *E. coli*. It could be visually observed from the expression of the four clones in *E. coli* that two produced orange light, one produced yellow light, and one produced yellow-green light.

In order to ascertain whether other colours of bioluminescence could also be found in the library, it was rescreened for other full-length cDNA clones. The rescreening was done by a different method designed to identify luminescent activity directly. Five aliquots of the original library were amplified, then transformed *en bloc* into expression plasmids. As in the case of eukaryotic cell expressing the firefly luciferase (see above), bioluminescence can be initiated in *E. coli* expressing luciferase by the addition of luciferin to the media (Wood and DeLuca, 1987). In bacteria, the diffusion of luciferin through the membranes can be facilitated by reducing the pH of the media to 5. Presumably this masks negative charges on the molecule, making it more hydrophobic and permeable to a lipid bilayer. By adding luciferin to bacterial colonies containing clones of the cDNA library, colonies able to express a functional luciferase were identified directly by their ability to darken X-ray film. Several bioluminescent colonies were isolated from each aliquot of the library, seven of which were identified as arising from independent cDNA clones. From two of the aliquots, two colonies could be judged as resulting from independent clones based on widely different intensities. The independence of these clones was later confirmed by restriction mapping. From these clones, five emit yellow light, one emits orange light, and one emits a new colour, green.

Western blot analysis was performed to confirm that full-length click beetle luciferases were being expressed in the *E. coli*. Despite the fact that some of these clones were clearly visualized by anti-firefly luciferase antibody during the library screening, we were unable to detect the gene products in blots made directly with *E. coli* lysates. This is the result of both a low level of gene expression, and a weak cross-reactivity with the antibody. The expression of luminescence was increased by transferring the cDNA clones to a plasmid vector which incorporated a *tac* promotor (Fig. 3). A lysate from *E. coli* expressing the green-emitting luciferase from the *tac* vector further required partial purification to be detectable in a blot. The blot revealed a single band, cross-reactive with firefly luciferase, which comigrates with the native click beetle luciferase (Fig. 4). DNA sequence analysis was later performed

Figure 4. Western blot showing the expression of click beetle luciferase in *E. coli*. Lane 1: partially purified extract of *E. coli* expressing the green-emitting luciferase. Lane 2: extract of click beetle light organ. Lane 3: purified firefly luciferase. Luciferases were detected with anti-firefly luciferase

for one clone of each colour. This confirmed that each cDNA contained an open reading frame which could code for a protein whose N-terminus corresponded to the N-terminus of firefly luciferase. Thus, as has been achieved previously with the firefly luciferase, the click beetle luciferases can be produced in *E. coli* as full-length and enzymatically active enzymes.

BIOLUMINESCENCE SPECTRA OF CLICK BEETLE LUCIFERASES

Spectrographic analysis was performed on the bioluminescence emitted from *E. coli* expressing the various cDNA clones. Bioluminescence was induced from whole cells by the same method used previously in the screening of bacterial colonies for luminescence. Cells producing luciferase from the *tac* vector yielded sufficient light intensity, upon addition of luciferin to the media, to allow spectral measurements. These measurements verified the visual observation that the eleven clones can be sorted into four groups based on the colour of light emitted. For each of the colours, the spectrum is a single peak qualitatively similar to the spectra of native click beetle luciferase (Seliger *et al.*, 1964). When the spectra of the four colours are superimposed, they show a remarkable pattern of four similarly shaped peaks that are nearly evenly spaced (Fig. 5). The wavelengths of maximum intensity are 546 nm for green, 560 nm for yellow-green, 578 nm for yellow, and 594 nm for orange.

Spectra were also measured from lysates of the *E. coli* after partial purification (Wood *et al.*, 1988a) (Fig. 6). Bioluminescence was elicited from the lysates by diluting them 100-fold into a reaction mixture ranging in pH from 6 to 10. For pHs 6.0, 7.0, and 8.0, the reaction mixture was buffered with 50 mmol/l MES/50 mmol/l MOPS/50 mmol/l Tricine. For pHs 8.0, 9.0, and 10.0 it was buffered with 50 mmol/l Tricine/50 mmol/l CHES. Also in the reaction mixture were 5 mmol/l $MgSO_4$/1 mmol/l EDTA/0.1 mmol/l luciferin/1.5 mmol/l ATP/1 mmol/l NaF/0.2 mg/ml BSA/10% glycerol. (NaF was found to simplify the kinetics of the decay of luminescence, which simplified the analysis of the spectral data. It does not affect the spectral distribution. It is not known whether it affects the activity of the click beetle luciferase directly, or whether it is due to an interaction with other components of the lysate. It has no effect on the purified firefly luciferase.) For the click beetle luciferases from each of the four colours, the spectra measured from whole cells matched that of the lysates at pH 6.0 and pH 7.0. Also, for each of the luciferases, the spectra shifted towards longer wavelengths at pH above 9.0. This shift was largest for the green-emitting luciferase, less for the yellow-emitting luciferase, and the least for the yellow-green and orange-emitting luciferase. At pH 8.0, this shift is virtually undetectable for the yellow-green and orange-emitting luciferases. For the green and yellow-emitting luciferases, the shift at pH 8.0 can be detected as a slight widening of the spectral peak, but the position of the maxima is unchanged.

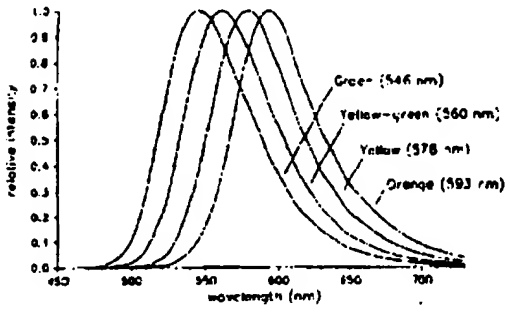

Figure 5. Spectra of bioluminescence emitted from *E. coli* cells containing the click beetle luciferases. The intensity maximum for each spectrum has been normalized.

This pH response of the click beetle luciferases is in contrast with that of the firefly luciferase. As stated above, the spectrum of firefly luciferase shifts to longer wavelengths at low pH (Fig. 7). In the pH range of 8.0 to 10.0, the enzyme emits its characteristic yellow-green light. The spectrum shifts towards longer wavelengths at pH 7.0, and at pH 6.0 is generated almost completely from a red-light emitting form of the enzyme. This shift is much larger than is seen with the click beetle luciferases. At pH 7.0, where the spectrum of firefly luciferase is a mixture of yellow-green and red-emitting forms of the enzyme, a difference is apparent between the enzyme purified from fireflies and that produced in *E. coli* (Fig. 7). With the luciferase from *E. coli*, the red component of the spectrum is much less than for the purified native enzyme. In addition, as the light output of the reaction decays, the two components of the spectrum do not decay at the

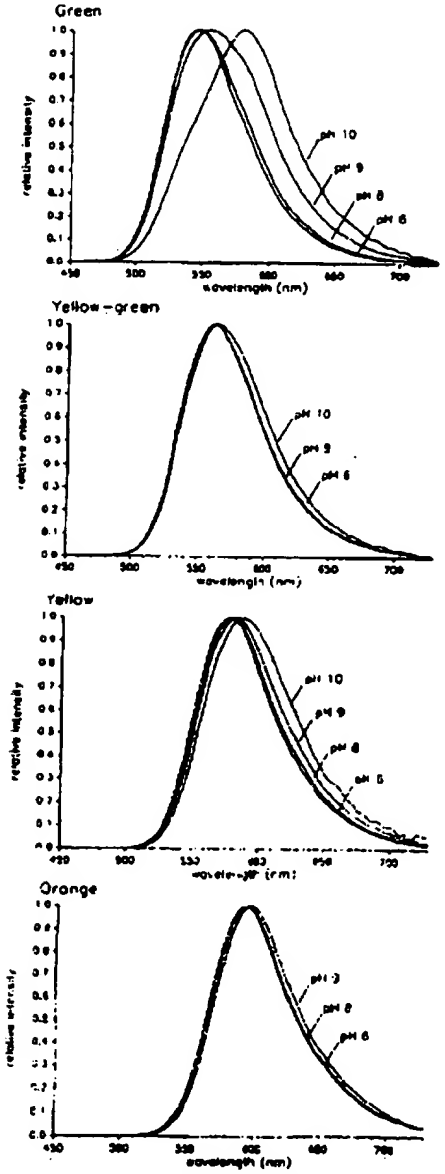

Figure 6. Spectra measured from partially purified lysates of *E. coli* expressing the click beetle luciferases. The intensity maximum for each spectrum has been normalized. The colour emitted by each luciferase at neutral pH is indicated in the corner of each plot. Spectra shown for pH 6.0 and 8.0 were measured in MES/MOPS/Tricine buffer. Spectra shown for pH 9.0 and 10.0 were measured in Tricine/CHES buffer. At pH 8.0, the spectrum measured in MES/MOPS/Tricine was identical to that measured in Tricine/CHES

same rate. The decay rate of these two components is the same rate in the spectrum of the native luciferase. The spectra of the luciferase produced in *E. coli* are of samples that are only partially purified by the method stated above. It can be shown that the differences between this and the native luciferase are not due to intrinsic differences in the enzymes themselves, but instead arise from the effects of the other components in the bacterial lysate. If the native luciferase is mixed with a lysate prepared from *E. coli* which does not contain a luciferase cDNA clone, the spectrum of the mixture is the same as that of lysates containing the luciferase produced in *E. coli*.

These observations reveal two aspects of the effects of an *E. coli* lysate on the spectrum of firefly luciferase. One is that the lysate contains a component that causes luciferase to resist the effects of pH on its spectrum. The other feature is

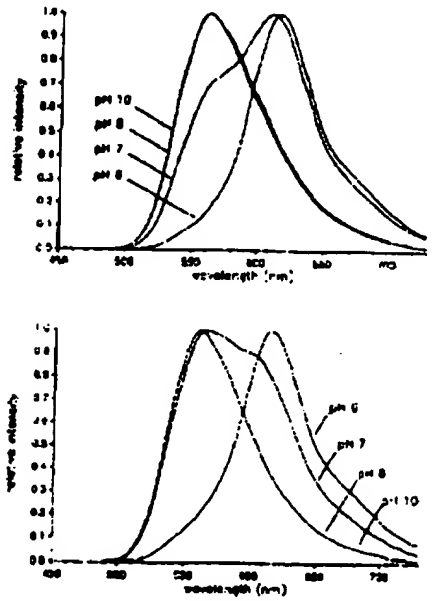

Figure 7. Spectra measured from purified native firefly luciferase (upper plot) and from partially purified lysates of *E. coli* expressing the firefly luciferase (lower plot). The intensity maximum for each spectrum has been normalized. Spectra shown for pH 6.0 and 7.0 were measured in MES/MOPS/Tricine buffer. Spectra shown for pH 8.0 and 10.0 were measured in Tricine/CHES buffer. At pH 8.0, the spectrum measured in MES/MOPS/Tricine had a slightly greater contribution from the red component than that measured in Tricine/CHES

that firefly luciferase, in the presence of the lysate, is in at least two different forms distinguished both by the colour of light emitted, and by different decay rates of the light output. These two features may indicate a single phenomena. That is, a factor in the lysates may be stabilizing some of the luciferase molecules both to destabilizing effects of low pH, and to the temporal loss of enzymatic activity. The presence of the bacterial lysate does not appear to affect the spectral distribution of each of the components of the firefly luciferase spectrum, just their relative contribution to the total spectrum. While this effect is most evident when the spectrum is measured at pH 7.0, it is also evident at pHs 6.0 and 8.0. In these cases, however, the differences are slight since the spectrum consists almost entirely of a single component. Extrapolation of these results to the spectra of the click beetle luciferases incidate that their spectral distributions in the pH range of 6 to 8 are probably not affected by the lysate. This is true since the spectrum of these luciferases is apparently a single component in this pH range. But the pH required to shift the spectra to longer wavelength is potentially different than what would be expected for purified enzymes. However, the spectra of the green-emitting click beetle luciferase at pH 9.0 or 10.0, which also consists of two components, did not reveal the nonuniform decay rate evident with the firefly luciferase at pH 7.0.

SEQUENCE COMPARISON OF CLICK BEETLE AND FIREFLY LUCIFERASES

Our inability to demonstrate cross-hybridization of their corresponding nucleic acid in Southern blots suggested that a significant degree of evolutionary divergence had occurred between the firefly and click beetle luciferases. Sequence analysis of the click beetle cDNA clones has confirmed this. For a direct comparison with the firefly luciferase, the yellow-green-emitting click beetle luciferase was used since its spectral maximum is at nearly the same wavelength. The cDNA encoding this luciferase contains an open reading frame corresponding to 543 amino acids. This is seven amino acids less than that found with the firefly luciferase cDNA. Alignment of the amino acid sequences, deduced from the cDNA sequences, reveals a 47% identity between the two luciferases (Fig. 8). The difference in the number of amino acids between the sequences is mostly accounted for by six gaps in the sequence alignment. These gaps are small, being one or two amino acid in length and, for some, their exact position is somewhat arbitrary.

Throughout the alignment there are no regions of especially high sequence similarity. Thus there is no indication of which regions may have been conserved owing to catalytic or structural constraints on the enzymes. An exception to this is in the last three amino acids which, for the firefly luciferase, have been shown to be necessary for translocation into peroxisomes (Gould and Subramani, 1988b, 1988c). Given the close functional similarity of these enzymes, it is almost certain that the click beetle luciferases are also located in peroxisomes. The hydropathy plots of firefly and click beetle luciferase shown some similarities, but overall appear to be quite different (Fig. 9) (Kyte and Doolittle, 1982). The most apparent similarity is the three large hydrophobic regions found in both luciferases. Other regions of hydrophobicity and hydrophilicity can be found in common between the luciferases, but they are largely obscured in as many differences.

In contrast to the low degree of similarity between the firefly and click beetle luciferases, the similarity between the various click beetle luciferases is very high. Between the luciferases which are capable of emitting different colours, the amino acid sequences are from 95% to 99% identical (Wood *et al.*, 1988b). Since the only difference between these luciferases are the amino acid sequences, the determinants of colour must be found in the relatively few differences between the sequences. We have begun to examine exactly which of the amino acids can affect the colour of light, and have found that not all of the differences between the clones are effective. In some cases, the amino acid determinants of colour may be as few as two or three. This work will be presented elsewhere.

USE OF CLICK BEETLE LUCIFERASES AS GENETIC REPORTERS

It appears that the click beetle luciferases will have all the advantages of the firefly luciferase in their application as reporters of genetic activity. Advantages such as the sensitivity with which they can be detected, or the ability to detect them

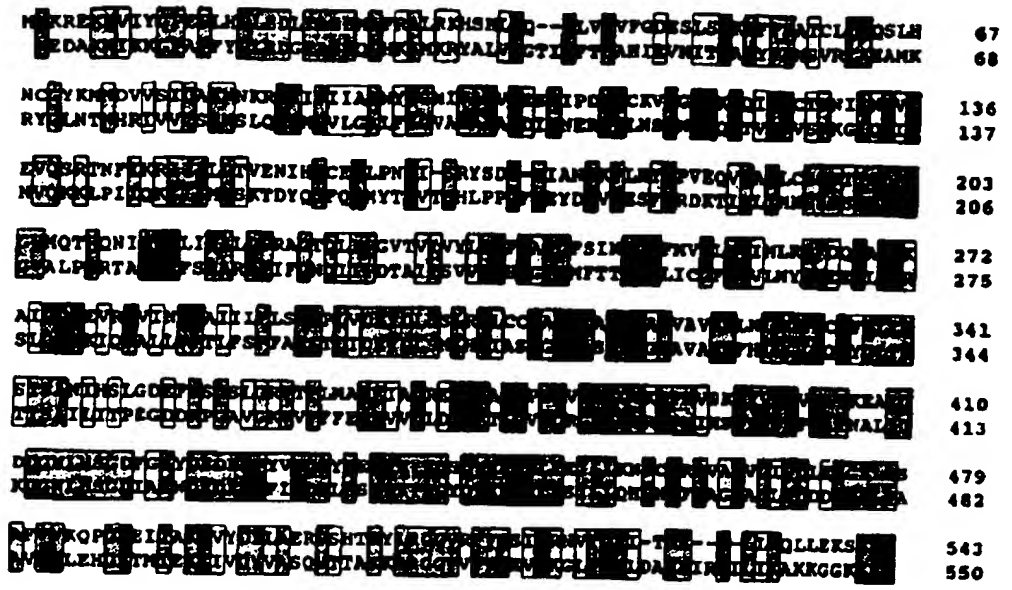

Figure 8. Alignment of the yellow-green-emitting click beetle luciferase (top line) and the firefly luciferase (bottom line). Regions where the aligned amino acids are identical are indicated by dark grey boxes; regions where the amino acids are similar, but not identical, are indicated by light grey boxes. Gaps in the alignment are indicated by hyphens. Numbers on the right indicate the position of the amino acid at the end of each line

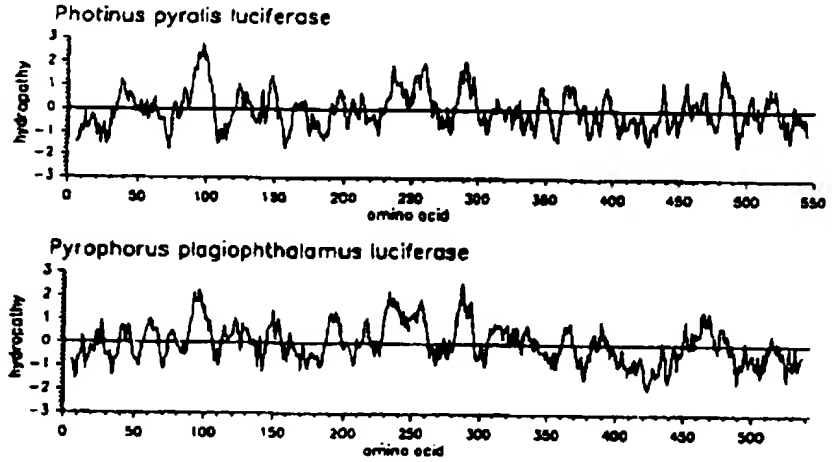

Figure 9. Hydropathy plot of the click beetle (lower plot) and firefly (upper plot) luciferases. Plots were calculated by the Kyte–Doolittle method with a window of 11

in living cells, are evident. Other features of the luciferases, such as their specific activity, or the linearity of their assay with respect to enzyme concentration, have yet to be established. We are at present improving the expression of these luciferases in *E. coli* to provide a source of enzyme for better characterization. It may be expected with the large difference in sequence between the click beetle and firefly luciferases, that these luciferases also have significant differences in their chemical properties as well. This is supported by the dramatic difference in the response of their spectra to changes in pH. By qualitative observation, temperatures above 40 °C or the presence of Zn^{2+} do not cause changes in the spectra of the click beetle luciferases. As noted above, these conditions will cause the firefly luciferase to emit red light. In fact, the temperature optimum for the click beetle luciferases may be higher than for the firefly luciferase. Other initial experiments suggest that the click beetle luciferases may be more resistant to denaturation by charged detergents, or activation by neutral detergents (Kricka and DeLuca, 1982). Collectively, these observations suggest that the activity of the click beetle luciferases may be less sensitive to environmental conditions. However, these conclusions are tentative since they were made from luciferases in the presence of other components of the *E. coli* lysate.

A novel feature of the click beetle clones is the ability to distinguish between them by the colour of light emitted. This may make them particularly useful as genetic reporters where multiple reporters are desirable. Because their respective sequences differ by only a few amino acids, characteristics of their expression in exogenous hosts should also differ little. The differences in the colour of light would normally have no effect on the hosts, but regardless, expression of a luciferase reporter gene is generally done in the absence of the luciferin substrate. Thus there is no luminescent activity until the actual time of the luciferase assay. The spectral distribution of the luciferases are rather broad which would limit the ability to distinguish each luciferase in a mixture if their respective amounts vary widely. The greatest distinction can be made between the green and orange-emitting clones, which should be distinguishable in a luminometer with the use of optical cut-off filters. From calculations based on the overlap of their spectra alone, and assuming a coefficient of variation of 4% in the assay of luminescence, this method should allow the detection of one of the luciferases in the presence of a 25-fold excess of the other. Since the colours of these luciferases are not easily altered by pH or temperature, it should be possible to distinguish these luciferases *in vivo* as well as *in vitro*. This type of dual reporter gene system would allow one to monitor different promoters within a single host, or to follow different populations of cells simultaneously, each labelled with a different luciferase. The structural similarity of the luciferases increases confidence that differential effects noted in an experiment are properties of the system being observed, and are not artefacts due to individual peculiarities of the reporter genes themselves.

SUMMARY

Firefly luciferase has been used as a tool of scientific investigation for over two decades because of the high sensitivity with which its enzymatic activity can be assayed. With the advent of techniques in nucleic acid manipulations, it has found its newest area of application as a reporter of genetic activity within living cells. In addition to high sensitivity, its assay is rapid and does not require complex procedures or precautions. In comparison to the CAT assay, firefly luciferase has been shown to be well suited as a genetic reporter. But, whereas previously firefly luciferase was the epitome of beetle luciferases because of its availability, cloning techniques have made feasible the study of other luciferases of this type. Some of these luciferases may have additional features enhancing their use as reporters, or in other applications. Our recent cloning of several luciferases from a bioluminescent click beetle substantiates this possibility. These luciferases are unique in the ability to produce bioluminescence of several different colours. In addition, the sequence of these luciferases is considerably different from that of the firefly luciferase, suggesting that other chemical properties of these enzymes will be different. One area where such differences are apparent is in the response of the bioluminescence spectra to changes in pH. We are currently investigating other properties of these new luciferases to better understand their general nature and to determine their suitability in applications.

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JOURNAL OF BIOLUMINESCENCE AND CHEMILUMINESCENCE VOL 5 107–114 (1990)

Luc Genes: Introduction of Colour into Bioluminescence Assays

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Luminescence assays are generally based on measurements of light intensity alone. Inclusion of colour as an additional parameter of the assay could increase the information content. Colour variation in luminescence is particularly prevalent among beetle luciferases. To study the relationship between enzyme structure and colour, luciferases from a Jamaican click beetle were examined as a model system. These luciferases emit light ranging from green to orange, though their amino acid sequences differ by less than 5%. Through mutation of their respective cDNA clones, the amino acids responsible for the colour variation were identified. These specific amino acids are few, and they act upon colour independently with respect to the enzyme structure. Analysis of their effects indicates that the potential for colour variation among beetle luciferases is greater than is evident among the click beetle luciferase. Because of the subtle changes of enzyme structure that effect colour, luciferases that emit different colours may be useful as paired genetic reporters. They should interact equivalently with the intracellular environment of a host, but could be distinguished by colour in their assay. Such paired reporters could be used to observed simultaneous events, or to provide internal control for luminescence measurements.

Keywords: Firefly luciferase; click beetle luciferases; reporter genes; colour variation

INTRODUCTION

The production of light by enzymatic catalysis offers unique opportunities for probing biochemical processes. This is because of the high energy density of photons and their unusual presence in a biochemical milieu. In applications of bioluminescence, the chemistries of luminous bacteria and beetles have been dominant. Although the mechanisms of these two systems are entirely different, they both are amenable to manipulations: the enzymes are reasonably stable and easily purified, the luciferins are available by chemical synthesis, and the other substrates are readily obtainable in pure form.

Luminescence as a biochemical guage is based on correlating light with a limiting component of the enzymatic reaction—changes in concentration of the limiting component cause proportionate variation in light emission. Initial applications of luminescence used cofactors as the limiting components. For example, firefly luciferase has been widely used to measure ATP. Similarly, bacterial luciferase coupled to an oxidoreductase has been used to measure NADH. With coupling to other enzymes, these luciferases have also been used to measure other biochemical molecules (McElroy and DeLuca, 1983).

Recently, a new class of luminescence applications has arisen where the enzyme is the limiting component. In these applications, light emission is linked to events associated with gene regulation

0884-3996/90/020107-08$05.00

Received 6 December 1989

and protein metabolism. This was made possible with the cloning of genes that code the luciferases (Cohn *et al.*, 1983; de Wet *et al.*, 1985). These genes can be introduced into living cells, or reconstituted enzyme system. so that the synthesis of luciferase is contingent upon the kinetics of gene expression. With excess substrates, luminescence is proportional to the concentration of newly synthesized enzyme.

Because of the prevalence of research in molecular genetics, applications of bioluminescence are most auspicious in this area. In eukaryotic systems especially, the use of firefly luciferase has been notable. This monomeric enzyme, evolved in a eukaryotic host, requires no post-translational modifications for its catalytic activity. Under optimal conditions, it catalyses production of yellow-green light with exceptional efficiency (McElroy and DeLuca, 1985: Seliger and McElroy, 1960).

The general suitability of this luciferase as a genetic reporter has made it useful in a variety of experimental designs. Most commonly it has been used in examining the DNA structure of genetic regulatory elements (Economou *et al.*, 1989; Hudson *et al.*, 1989; van Zonneveld *et al.*, 1988). Some studies have used this luciferase to investigate other proteins that influence gene transcription (Mellon *et al.*, 1989; Waterman *et al.*, 1988). Also studied have been effects of mRNA structure on protein synthesis (Malone *et al.*, 1989; Baughman and Howell, 1988), and relative rates of intracellular protein recycling (Nguyen *et al.*, 1989). The firefly luciferase has in some instances been used to delineate genetic events in multicellular organisms (Rodriguez *et al.*, 1989; Ow *et al.*, 1986).

Common to these luminescence applications is that measurements are made of light intensity alone. However, this is only one mode by which light can carry information. Another prominent property of light is its spectral distribution, i.e. the colour of light. If this property could be used in addition to intensity, it could add another dimension to the information transmitted by the luciferases. Each luciferase elicits a characteristic spectral distribution. Even within the distinct groups of beetle or bacterial luciferases there is variation of colour. Since the substrates within these groups do not differ, the colour variation must be due to differences in enzyme structures.

Colour variation is especially prominent among the bettle luciferases. A spectacular example of this variation occurs in a tropical click beetle from Jamaica, *Pyrophorus plagiophthalamus*. The beetle has two sets of light organs, a pair on the dorsal surface of the prothorax, and a single organ in a ventral cleft of the abdomen. Generally the dorsal pair emits green light, and the ventral organ emits yellow light. Hence, this is an unusual example of an organism that emits two different colours of light. Even more unusual is that variation in colour occurs between individuals of the population. The dorsal organ varies in colour from green to yellow-green, and the ventral organ varies from green to orange (Biggley *et al.*, 1967).

Because of the wide range of colours found in this single species, it was chosen as a model of colour variation among beetle luciferases. Research was begun to investigate the relationship between enzyme structure and the colour of luminescence. Results of the ongoing project have revealed some general aspect of this relationship. Substantial changes in colour can result from substitutions of single amino acids in the primary structures of the enzymes. These substitutions can occur at several different positions, and the effect of different substitutions act independently. A quantitative analysis of several substitutions has indicated that the potential for colour variation in beetle luciferases is much greater than the range of colours found in this particular beetle species. These results foretell the feasibility of using colour as an additional parameter in luminescence assays.

COLOUR VARIATION IN *P. PLAGIOPHTHALAMUS*

To study the luciferases of the Jamaican click beetle, the cloning techniques previously employed to clone the firefly luciferase were used (de Wet *et al.*, 1985). The luciferases of the ventral light organ were chosen for initial study because of their wider range of colour variation. Screening a cDNA library made from this organ, both for antigenic epitopes and for luminescence activity, resulted in 11 clones with complete coding regions. When expressed in *E. coli*, these clones can produce sufficient bioluminescence to be easily visible.

The clones are of four types determined by the colour of light elicited: green, yellow-green, yellow, and orange (Fig. 1). Among the seven clones that produce yellow light, or the three that produce orange light, the spectra are indistinguishable. Only one green and one yellow-green light-producing clone were obtained. As determined by the positions of the peak intensities, the range of colours

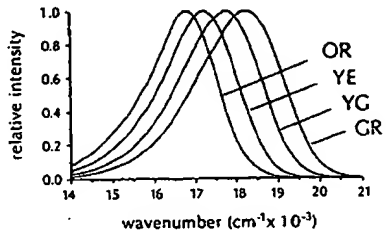

Figure 1. Luminescence spectra of the click beetle luciferases: GR, green; YG, yellow-green; YE, yellow; OR, orange. Intensity maxima are normalized for comparison

produced by the clones is the same as was measured from living beetles (Biggley *et al.*, 1967). However, there are colours displayed by living beetles that are not represented by the clones. Some of these colours may be the result of heterozygous beetles expressing a mixture of luciferases. Of course, the genes of some colours may not have been cloned yet.

The amino acid sequences of the click beetle luciferases were determined from the nucleic acid sequences of the clones. In paired comparisons these amino acid sequences are 96% to 99% identical, differing by 26 to 3 amino acids respectively. The comparisons reveal the genealogy of these enzymes, which shows that they have evolved in the order of their colours. Thus, luciferases of similar colour are of more similar sequence. Also, the most recently evolved luciferase emits orange light; the green-emitting luciferase is the oldest. So the sequences of the luciferases emitting orange and yellow are more similar than those of the green and yellow-green.

As noted above, the variation in colour must lie within differences of the enzyme structures. Since there is no evidence of post-translational modification in the luciferases, the differences should be evident within the amino acid sequences. To determine which (and how many) of the amino acids affect colour, mutants of the luciferases were made by modifying their cDNA clones. The mutants were made by two methods. Many were made simply by exchanging restriction fragments between the clones. The resulting new luciferases were named rearrangement hybrids. Other mutants were made using synthetic oligonucleotides for site-specific changes.

Because the rearrangement hybrids were made by swapping segments of genetic code, they often contain multiple amino acid substitutions. Notation to describe these substitutions is, for example, $R_{223},L_{238} \rightarrow E,V$. This depicts the substitution of arginine at position 223 and leucine at position 238 for glutamine and valine respectively. The resulting changes in the colour are reported in wave numbers instead of wavelength since wavenumbers are proportional to energy, an additive quantity. In the example, the substitutions cause a colour shift of $-520\ cm^{-1}$, from $17{,}760\ cm^{-1}$ of the parent luciferase to $17{,}240\ cm^{-1}$ of the progeny.

The results of studying several mutants show that colour differences among the yellow-green-, yellow-, and orange-emitting luciferases are due predominantly to three amino acid substitutions. The colour difference between the yellow- and orange-emitting luciferases is due entirely to $S_{247} \rightarrow G$. Approximately 90% of the colour difference between the yellow-green- and yellow-emitting luciferases is due to two substitutions, $R_{223} \rightarrow E$ and $L_{238} \rightarrow V$; the colour shift caused by $L_{238} \rightarrow V$ is about 1.3-fold greater than that of $R_{223} \rightarrow E$. The remaining 10% of colour difference is due to one or more substitutions of L_{41}, D_{226}, $V_{282}I_{283}$, V_{323}, $V_{389} \rightarrow I,E,IV,I,I$. Likewise, the amino acids affecting colour of the green-emitting luciferase have not yet been precisely determined because of the large number of sequence differences between this and the other luciferases.

From other mutants it is evident that the substitutions that affect colour do so regardless of the parent luciferase. For example, the $S_{247} \rightarrow G$ substitution, which causes the yellow-emitting luciferase to produce orange light, can be applied to the yellow-green-emitting luciferase. This causes an analogous shift of colour to yellow. Similarly, $E_{223} \rightarrow R$, which partially effects the shift from yellow to yellow-green, produces an analogous shift towards green when applied to the orange-emitting luciferase. Apparently, colour differences evolved in the click beetle luciferases by the cumulative effects of individual substitutions. That is, the change from yellow-green to orange requires the combined action of $R_{223} \rightarrow E$, $L_{238} \rightarrow V$, and $S_{247} \rightarrow G$ (with small contributions from other substitutions). Presumedly, this general scheme applied also to the green-emitting luciferase.

INDEPENDENCE OF SUBSTITUTIONS AFFECTING COLOUR

When amino acid substitutions in an enzyme act independently, the action of one substitution

should not affect the action of the other. As a corollary, the individual effects of the substitutions should be additive. In the opposite situation, extreme cooperativity, the action of one substitution is fully dependent on the other. Among the click beetle luciferases, the cumulative action of the substitutions that affect colour rules out extreme cooperativity. Superficially, however, the substitutions do not appear to act entirely independently.

For example, the rearrangement hybrid with substitutions E_{223}, $V_{238} \rightarrow$ R,L causes the colour of the yellow-emitting luciferase to shift 490 cm^{-1}. However, the combined effects of $E_{223} \rightarrow R$ and $V_{238} \rightarrow L$ applied to the yellow-emitting luciferase is a shift of 420 cm^{-1}. Thus, the individual affects appear to be 14% less than their combined affect. As another example, the substitutions $S_{247} \rightarrow G$ applied to the yellow-emitting luciferase causes a shift of $-430\ cm^{-1}$. However, when the same substitution is applied to the yellow-green-emitting luciferase, the resulting shift is $-580\ cm^{-1}$, 35% greater.

Close examination of the shifts caused by each type of substitution has revealed a consistent relationship with regard to the colour of the parent luciferase. Specifically, substitutions have a greater affect on colour when applied to luciferases of greater wavenumber. This effect is shown quantitatively in a plot of shift magnitude *vs* shift position (Fig. 2). The magnitude is simply the absolute value of the difference in positions of spectral maxima between the parent and progeny luciferases, i.e. the shift without regard to sign. The positions of the shift was taken as the average of the position of spectral maxima for the parent and progeny luciferase. This was chosen instead of the position for the parent luciferase since, in theoretical considerations, the distinction between parent and progeny is arbitrary. Thus, by using the average as a measure of position, the choice of parent or progeny is mute.

It is apparent from the plot that the different types of substitutions define a converging set of trends. The substitutions (or groups of substitutions), whose resulting shift are shown as filled symbols in Fig. 2, respresent independent sets. The grouped substitutions, whose resulting shifts are shown as open symbols, include within themselves substitutions of the independent sets. Although these shifts do not result from independent substitutions, they are derived from independent measurements of rearrangement hybrids. Thus, they provide additional evidence for the trends.

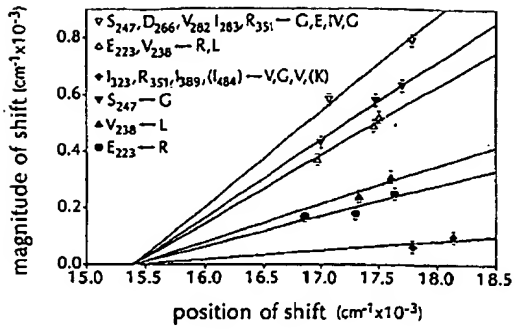

Figure 2. Relationship of shift magnitude to the position of the shift. Key to amino acid substitutions is shown on the left. Filled symbols show shifts caused by independent substitutions; open symbols show shifts resulting from combined sets of substitutions. Bars above and below each data point indicate one standard deviation of experimental error. See text for criteria of the axes and description of interpolated lines

Using linear least-squares analysis, a set of lines was determined for the shifts of each set of substitutions. Most of these lines cross the abscissa near 15,500 cm^{-1}. The lines drawn in Fig. 2 are an interpretation of the data. These lines converge to a single point on the abscissa. The inference is that there exists a unique minimum for the position of the luminescence spectrum. Effects of the substitutions are not able to exceed that minimum. That is, there is a limit to how red the luminescence can be and no alterations to the luciferase structure can result in light that is more red.

This seems reasonable with regard to the structure of the light-emitting molecule. Beetle luciferases catalyse light production by combining ATP and luciferin to form luciferyl-AMP, which is then oxidized to oxyluciferin. The oxyluciferin is formed in an eletronically excited state, and a photon is generated upon its transition to the ground state. The colour of luminescence is determined by the energy difference between the ground and excited states. This difference is influenced by interactions of the oxyluciferin with the enzyme structure. Therefore, colour can be affected by substitutions of specific amino acids. However, without changes to the bonding structure of oxyluciferin, there should exist a minimum possible energy difference between the ground and excited states. This minimum would impose a minimum of the energy of the emitted photon, i.e. the colour of light.

The lines in Fig. 2 were drawn to converge at 15,400 cm^{-1}. This value is the position of maximal

intensity for the luminescence generated by oxidation of luciferyl-AMP in aqueous buffer without enzyme (White *et al.*, 1971). It is the lowest wavenumber value measured for the luminescence of oxyluciferin under any conditions, with or without enzyme. It is known that the colour of luminescence elicited from non-enzymatic oxidation is dependent on the polarity of the solvent. Under less polar conditions, such as in DMSO, the spectrum of luminescence shifts to greater wavenumbers (White *et al.*, 1971). Water is a highly polar medium and may provide conditions for luminescence of minimum energy.

Drawing the trends of spectral shifts as lines converging at 15,400 cm^{-1} is in good agreement with the data. each of the data points is near its respective line within one standard deviation of experimental error. Thus, the empirical data is in accord with the hypothesis of an energy minimum for the luminescence. Since this minimum was measured from a non-enzymatic reaction, it endorses the belief that this minimum is determined entirely by physical properties of oxyluciferin. Hence, where the lines converge in Fig. 2 should be independent of structural features particular to the click beetle luciferases. Moreover, colour changes caused by amino acid substitutions in any beetle luciferase should exhibit trends that converge to this same minimum value.

The apparent lack of independence noted above between colour shifts is evident in the slopes of the lines in Fig. 2. If the shifts had displayed the sense of independence describe at the beginning of this section, then the slopes would have to be zero. That is, regardless of the colour of the parent luciferase, the substitutions would cause the same magnitudes of shift. Yet, slopes of zero would imply no minimum to the energy of luminescence. Thus, the interdependence between the effects of substitutions imposed by the slopes is due to physical limitations of the substrate.

Within this constraint, however, the effects of the substitutions behave completely additively. For example, calculated from the lines of Fig. 2, $E_{223} \rightarrow$ R applied to the yellow-emitting luciferase would result in a shift of 204 cm^{-1}. Applying $V_{238} \rightarrow$ L to the resulting hypothetical luciferase would result in an additional 288 cm^{-1}. The sum of these shifts, 492 cm^{-1}, is only 1% less than the expected value of E_{223}, $V_{238} \rightarrow$ R,L applied to the yellow-emitting luciferase, 498 $c,^{-1}$. Similarly, a shift caused by S_{247}, D_{266}, $V_{282}I_{283}$,$R_{351} \rightarrow$ G,E,IV,G is equal to the sum of its component substitutions. Because the trends of Fig. 2 are described by lines, additive relationships demonstrated for one luciferase are the same when applied to other luciferases. Also, it follows that the order in which the substitutions are considered is unimportant.

Therefore, respective to the slopes of the lines, the effects of the substitutions act fully independently. In equivalent terms, the substitutions are independent with regard to their action on the substrate; the apparent interdependence of colour shifts can be attributed to properties of oxyluciferin. There is no evidence of dependent relationships mediated by the structures of the enzymes. That is, there are unlikely to be any interactions within the enzyme structures between the amino acids at the positions of the substitutions.

POTENTIAL FOR COLOUR VARIATION AMONG BEETLE LUCIFERASES

The colours of luminescence emitted by the Jamaican click beetle define nearly the full range of colours found in all luminous beetles (Lall *et al.*, 1980). Yet, the trends depicted in Fig. 2 suggest a potential for colour variation in beetle luciferases that is much greater. The range spanned by the click beetle luciferases is 1400 cm^{-1}. If the lower end of this range were extended to its theoretical limit, the full range would double to 2800 cm^{-1}. Furthermore, there is no indication in Fig. 2 of an upper limit to the possible range. Certainly an upper limit exists owing to conservation of energy in the luminescent reaction. However, it is unknown what further considerations could impose a more strcit upper limit.

The ability of beetle luciferases to support redder colours of luminescence is evident in the luminescence of the firefly luciferase (*P. pyralis*). Though this enzyme normally emits yellow-green light, under several conditions it emits red light of 16,160 cm^{-1} (McElroy and DeLuca, 1985). Some of these conditions are pH below 7, temperature above 30°C, and the presence of heavy metals such as Hg^{2+}. Chemical modification to the enzyme can also result in red luminescence (Alter and DeLuca, 1986). This red colour extends the range of enzymatic luminescence by 50% over that of the click beetle luciferases alone.

Nature has also provided one known example of red beetle luminescence in a rare species called *Phrixothrix*. This larviform beetle of South America has two rows of light organs that emit yellow-

green light, and a pair near the head that emit red light. Although the spectra of this red has not been measured, it is of much lower wavenumber than the orange of the click beetle. Thus, red luminescence is also possible through the mechanism of natural evolution. Since post-translational modifications that affect colour are not found in either the firefly or click beetle luciferases, the evolution of *Phrixothrix* luciferase is probably also mediated by modfications to the amino acid sequence.

So why do virtually all species of luminous beetles emit light in the limited range of green to yellow? The above discussion indicates that the enzyme is capable of supporting a much larger range. The reason may be in the 'motive' of beetle luminescence. The system has evolved to maximize communication between beetles, i.e. to maximize visibility. An essential aspect of this is the way in which colour interacts with the environment. For example, green is the colour of maximum reflectivity for foliage. Also, measurements of ambient light at dusk in a foliated area reveal a minimum near yellow (Seliger *et al.*, 1982b). The colours of beetle luminescence may be partially dictated by these environmental parameters, depending on the behavioural characteristics of the species. Evidence for this has been documented for firefly luminescence (Seliger *et al.*, 1982a,b).

However, the needs of beetle communication do not necessarily equal the needs of genetic research. In applications to utilize luciferase of different colours, a wide range would be more useful. Further study of colour variation in beetle luciferases should allow development of synthetically modified enzymes that elicit colours not found in nature. This could accord access to the full colour potential of this luciferase system. The lesson of the click beetle luciferases is that such modifications may encompass only substitutions of independently acting amino acids. Moreover, this natural example shows that there may be many candidates for such substitutions.

This follows from the relatively recent evolutionary history that brought about the different colours of the click beetle. Evolution is unable plan in its course; it can only operate by selection of randomly provided mutations. Yet, in the 26 amino acids that distinquish the four click beetle luciferases, more then four affect colour. Thus, by trial-and-error evolution, acceptable candidates for colour variation were rapidly found. It can be inferred that little of the mutagenic potential of these enzymes was tested by this process from comparison with the firefly luciferase. The luciferases from these two beetle species differ in amino acid sequence by 51%. Thus, amino acid substitutions are potentially acceptable at 275 positions. For each position, several different amino acids may suffice. In brief, the beetle luciferases appear to hold much potential for modification and much potential for variation in colour of luminescence.

APPLICATION OF COLOUR IN LUMINESCENCE ASSAYS

In luminescence assays, light intensity is the signal that conveys information of biochemical events. With the use of two luciferases that emit light of different colours, it should be possible to provide two signals simultaneously. Beetle luciferases offer the potential of providing such a two-coloured system. This probably would be most useful in applications associated with molecular genetics. This is because applications utilizing two colours would generally depend on the enzymes as the limiting components of the assay. The concentration of the enzymes must be the source of the signals since the distinction of colour lies in the enzyme structures, Assays based on a substrate as the limiting component, ATP for example, would not benefit because the enzymes of both colours would generate signals dependent on the same condition. Thus, additional information could not be gained with the second colour.

The obvious use of two colours would be for simultaneous detection of two different events. This would be especially useful when the events are coordinated. An example could be the transcriptional activities of promoters regulated by a common mechanism. Moreover, the promoters need not be of a single host, such as with regulation mediating symbiotic or parasitic relationships. The luciferases also need not be used for quantitative measurements, but merely as markers for two populations. For instance, populations of a colony-forming organism could be identified visually by their colour of luminescence. The genes coding the luciferases may also offer a method of detecting genetic recombination events, depending on the positions of the colour-determining nucleic acid substitutions.

Another general use of two colours would be for provisions of an internal control in luminescence measurements. Precision in genetic measurements can be important, especially in eukaryotic hosts

where differences of two- to three-fold are significant. Internal controls are often needed to compensate for uncontrollable variables. A common example is in experiments where DNA is introduced into cells for measurements of transient gene expression. To compensate for variation in the efficiency of transfection, a second genetic reporter is sometimes included (Dirks *et al.*, 1989; Day and Maurer, 1989).

After a gene is introduced into a cell, there are other potential variables of gene expression, such as rates of translation and protein stability. These variables that concern the behaviour of a reporter within a cell are more difficult to compensate for by using a second reporter. The problem is that different reporters can behave quite differently in a common host. For instance, comparisons of luciferase to another commonly used reporter, chloramphenicol acetyl transferase (CAT), reveal substantially different kinetics of expression (Maxwell and Maxwell, 1988). As expected, the structures of these dissimilar proteins interact differently with the complex metabolism of the host.

The ideal solution would be to use reporters whose structures are identical, yet could be distinguished in their assay. Herin could be the major advantage of beetle luciferases. Since only a few amino acid substitutions are needed to alter colour, the overall structures of the reporters could be virtually identical. Hence, there would be little to allow discrimination of their interactions with an experimental host. That is, the host could not differentiate one reporter from the other. Upon assay, however, distinction between the reporters would be made by their colours of luminescence. The similarity between the luciferases would be especially prominent if the distinguishing amino acids were internal to the protein structures. This may be the case since the amino acids that affect colour are likely to be close to the luciferin binding site.

Normally, experimental controls are implemented in genetic experiments through comparisons of a test population with a control population. Inclusion of an internal control would be most useful when inter-experimental variation is large, or replica experiments are difficult to obtain. For instance, replica populations could be difficult to achieve when the experimental host is not derived from a stable clonal source. In this circumstance, comparisons between test and control populations would be difficult. However, an internal control would allow for simultaneous comparisons of a test and control within an experiment. The closely matched structures of beetle luciferases could provide a means for internal control is experiments that utilize reporters. The light intensity of one colour would serve as the test signal, and the other the control signal. An example in which this may be useful is in measurements of transgenic organisms. Even though the hosts in these experiments may come from a clonal stock, acivity of exogenous genes inserted into their chromosomes can be strongly position-dependent.

An especially promising attribute of beetle luciferases as genetic reporters is the ability to detect their activity from within living cells. Two substrates of the luminescent reaction, ATP and O_2, are available in the cellular interior. The third substrate, luciferin, can gain access to the interior by diffusion through the membrane. Thus, in cells expressing luciferase, a luminescent signal can be generated for external detection. Because photons are created at the instant of catalysis and do not accumulate, the signal is a 'real-time' indicator of the intracellular luciferase concentration. However, quantitative measurements in living cells can be obscured by several variables. For instance, the enzyme may not be the limiting component of the assay in the intracellular environment since the availability of the other substrates could be limiting. Thus, changes in light intensity could reflect variations in any of the components.

Other factors, such as those described previously, also may conceivably affect intracellular luminescence. Use of two beetle luciferases to provide internal control could compensate for these influences. The test signal of one luciferase would be coupled to the gene of interest, and the control signal coupled to a reference gene. A suitable reference gene could be one of constitutive activity, a so-called 'housekeeping' gene. Measurements would be made not of absolute light intensity of the reporter signal, but by the relative intensity of the test signal compared with the control signal. Conditions that would affect both signals, such as changes in concentration of accessible internal substrates, could therefore be compensated for. By this method of measurement, indications of genetic regulation could be made with direct reference to the baseline genetic activity of the host.

In detail and variation, there doubtless are many ways in which beetle luciferases of different colours could be useful. The general suitability of a single luciferase as a genetic reporter is already amply demonstrated. Since introduction of this applica-

tion, each year has borne increased numbers of citations of its use in the scientific literature. The additional potential offered by luciferases that emit different colours lies in the subtlety of their structural differences. Such a matched pair of reporters, with the sensitivity and versatility of the luciferases, has not been manifested by other systems. Thus, the potential capabilities of beetle luciferases may not only improve current methods of assay, but in addition may endorse new methods. The possibilities presented here are based on our recent knowledge of the bettle luciferases, but further research will be needed to test the limits of these possibilities.

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The BIOCHEMICAL JOURNAL

Volume 279 1991

Editorial Board

Biochem. J. (1991) 279, 727–732 (Printed in Great Britain)

Engineering a bioluminescent indicator for cyclic AMP-dependent protein kinase

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cDNA coding for the luciferase in the firefly *Photinus pyralis* was amplified *in vitro* to generate cyclic AMP-dependent protein kinase phosphorylation sites. The DNA was transcribed and translated to generate light-emitting protein. A valine at position 217 was mutated to arginine to generate a site RRFS and the heptapeptide kemptide, the phosphorylation site of the porcine pyruvate kinase, was added at the *N*- or *C*-terminus of the luciferase. The proteins carrying phosphorylation sites were characterized for their specific activity, pI, effect of pH on the colour of the light emitted and effect of the catalytic subunit of protein kinase A in the presence of ATP. Only one of the recombinant proteins (RRFS) was significantly different from wild-type luciferase. The RRFS mutant had a lower specific activity, lower pH optimum, emitted greener light at low pH and when phosphorylated it decreased its activity by up to 80%. This latter effect was reversed by phosphatase. This recombinant protein is a good candidate to measure for the first time cyclic AMP-dependent phosphorylation in live cells.

INTRODUCTION

A universal feature of eukaryotic cells is the ability of physiological agonists, such as hormones, growth factors and neurotransmitters, components of the body's defence system, non-host antigens and other pathogens and drugs, to interact with the plasma membrane and trigger molecular events within the cell. These agents initiate a molecular sequence that starts with the generation of an intracellular signal, such as Ca^{2+}, cyclic AMP, inositol trisphosphate or diacylglycerol, and ends with a physiological or pathological event in the cell (Campbell, 1983; Berridge & Irvine, 1989). These events include movement, secretion, transformation, division, defence and death. The timing and magnitude of the end response in each cell depends on the timing and location of both the intracellular signals and the covalent modifications they induce. A particular cell will only undergo an end response if the right sequence of molecular thresholds has occurred (Campbell, 1983, 1988, 1990).

Measurement and imaging of intracellular Ca^{2+} using fluorescent and bioluminescent indicators (Campbell, 1983; Cobbold & Rink, 1987) has established that one explanation for gross heterogeneity in individual cell responses is variation in the timing and the location of the intracellular Ca^{2+} signal. In neutrophils, for example, four subpopulations have been defined, including one group showing no response at all (Hallett *et al.*, 1990; Davies *et al.*, 1991). A major problem in elucidating the molecular basis of heterogeneity within a cell population is the lack of a method for measuring and manipulating covalent modification of proteins in live cells. The purpose of the work reported here was to engineer cyclic AMP-dependent protein kinase phosphorylation sites into firefly luciferase, such that a change in colour and/or light intensity occurred after phosphorylation and dephosphorylation (Campbell, 1989).

Benzothiazole luciferases occur only in luminous beetles. They contain approx. 550 amino acids, and require ATP, Mg^{2+} and O_2, as well as a common luciferin, to generate light (Campbell, 1988). Just a few amino acid changes can cause the colour to shift from green to green–yellow, yellow or red (Wood *et al.*, 1989*a*,*b*). Recognition sites for protein kinase A (Cohen, 1988) have been added to α-interferon to allow high-specific-activity labelling for binding studies (Li *et al.*, 1989). In a previous study the heptapeptide kemptide (LRRASLG) (Zetterqvist *et al.*, 1976; Kemp *et al.*, 1977) was chemically coupled to extracted luciferase from the firefly *Photinus pyralis*. *Photinus* luciferase (*Photinus*-luciferin:oxygen 4-oxidoreductase; EC 1.13.12.7) emits yellow light with a peak intensity at 565 nm. The coupled kemptide shifted the colour of the light emitted to the red and phosphorylation shifted it even further (Jenkins *et al.*, 1990). Here PCR was used followed by transcription–translation *in vitro* (Sala-Newby *et al.*, 1990*a*,*b*) to change an amino acid sequence VRFS (217–220) (de Wet *et al.*, 1987) to RRFS, or to add kemptide to the *N*- or *C*-terminus of the protein.

MATERIALS AND METHODS

Materials

Oligonucleotide primers were prepared using an Applied Biosystems 381A DNA synthesizer and purified as 'trityl-on' oligonucleotides (100, 101) or 'trityl off' (105, 107, 108, 113, 114, T7-K). Their sequences were as follows:

100: TCATCGCTGAATACAGTTAC (3′ end antisense)
101: GGTAAAATGGAAGACGCCAAAAAC (5′ end sense)
105: CACCTAATACGACTCACTATAGGGAGAATGGAAGACGCCAAAAAC (5′ end antisense including the T7 promoter)
107: AGAACTGCCTGCCGCAGATACTCGCA (5′ end sense, underlined bases generate R codon)
108: TGCGAGAATCTGCGGCAGGCAGTTCT (3′ end antisense, underlined bases generate R codon)
113: CCTTGTCGACTTAGCCCAGGGAGGCCCGCCGCAGCAATTTGGACTTTCC (3′ end antisense with 21 bases coding for kemptide, a stop codon and a *Sall* restriction site)
114: GGCCTCCCTGGGCGAAGACGCCAAAAAC (5′ end sense, part of kemptide)
T7-K: CACCTAATACGACTCACTATAGGGAGAATGCTGCGGCGGGCCTCCCTGGGC (5′ end sense, clamp, T7 promoter and part of the coding sequence for kemptide)

Abbreviations used: CL count, chemiluminescence count; KNt, luciferase with kemptide at *N*-terminus; KCt, luciferase with kemptide at C-terminus.

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The coding sequence for firefly luciferase was isolated from a cDNA library (Sala-Newby *et al.*, 1990*a*,*b*). A 2400 bp *Sal*I fragment was used as the target for amplification. Amplitaq DNA polymerase was from Perkin–Elmer Ltd., U.K., T7 RNA polymerase was from Promega, nucleotides and Sephacryl S100 were from Pharmacia and Centricon 100 cartridges were from Amicon, U.K. [γ-^{32}P]ATP (10–50 Ci/mmol), [α-^{32}P]UTP (3000 Ci/mmol), stabilized [^{35}S]methionine ($\geq$ 1000 Ci/mmol), RNAase inhibitor and rabbit reticulocyte lysate (N90) were purchased from Amersham International plc. Restriction enzymes, alkaline phosphatase (24 units/μl) and luciferin were from Boehringer Corp. Low-protein-binding ultrafiltration units, Ultrafree MC, were from Millipore Corp. Protein kinase A inhibitor (P-3294) and kemptide were from Sigma Chemical Co. All other AnalaR-grade reagents were from Sigma Chemical Co. and BDH Chemicals. The catalytic subunit of cyclic AMP-dependent protein kinase was generously given by Dr. K. J. Murray of Smith Kline Beecham, Welwyn, Herts., U.K.

Preparation of DNA fragments

Addition of the T7 RNA polymerase promoter (TAATACGACTCACTATAGGGAGA) (Stoflet *et al.*, 1988) and the DNA sequence coding for kemptide (CTGCGGCG-GGGGTCCCTGGGC), as well as mutation of two bases within the luciferase cDNA, were carried out using PCR (Saiki *et al.*, 1988), as previously described (Sala-Newby *et al.*, 1990*b*). Firefly (*Photinus pyralis*) cDNA (4 ng/ml) was amplified in a solution containing 10 mM-Tris/HCl (pH 8.3), 50 mM-KCl, 2 mM-$MgCl_2$, 0.01 % (w/v) gelatin, 0.2 mM each of the four deoxynucleoside triphosphates, 0.5 μM of each oligonucleotide primer and 40 units of Amplitaq DNA polymerase/ml. The cycling reactions were carried out in a Perkin–Elmer thermal cycler. Each of the 25 cycles consisted of 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C plus a 5 s extension on each cycle. Klenow fragment of *Escherichia coli* DNA polymerase (40 units/ml) was added after the completion of the 25 cycles, and incubated for 30 min at 37 °C.

The final product was extracted once with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) and precipitated with 2 vol. of 7.5 M-ammonium acetate plus 2.5 vol. of ethanol. The DNA concentration was assessed visually from ethidium bromide-stained agarose gels by comparison with the bands of a standard DNA (Sambrook *et al.*, 1989).

Wild-type firefly luciferase DNA preceded by the T7 RNA polymerase promoter was prepared using oligonucleotide primers 100 and 105 (see under 'Materials'). The kemptide coding sequence was added to the 3′ end of the firefly cDNA using primers 105 and 113. The incorporation of the kemptide coding sequence at the 5′ end and the 2 bp change coding for the mutation V-217 → R were carried out in two stages (Higuchi, 1990). After the first amplification the primers were removed by filtration through Centricon 100 cartridges (Higuchi, 1990). The first stage of the introduction of kemptide at the *N*-terminus was carried out using oligonucleotide primers 114 and 100. The resulting DNA (4 ng/ml) was amplified for 25 cycles in the presence of oligonucleotide primers T7-K and 100 to produce the final product. The first stage of the preparation of the RRFS variant generated two fragments: 5′ end fragment (643 bp) was generated by amplification with oligonucleotide primers 101 and 108 and the 3′ end (1018 bp) with oligonucleotide primers 107 and 100. In the second stage the two fragments were mixed in equimolar amounts (2 μg of total DNA/ml), denatured (1 min at 94 °C) and allowed to reanneal by decreasing the temperature at 5.7 °C/min to 37 °C in amplification mixture containing primers 105 and 100 followed by 1 min extension at 72 °C. Eight to twelve cycles of amplification under normal conditions followed.

Formation of luciferase *in vitro*

PCR products (0.5–1.5 μg/25 μl of incubation mixture) were transcribed as previously described (Sala-Newby *et al.*, 1990*b*). The RNA capped using 0.5 mM-m^{7}G(5′)ppp(5′)G and 0.1 mM-GTP was precipitated twice with 0.2 vol. of 7.5 M-ammonium acetate and 2.5 vol. of ethanol. The RNA (1–100 ng) in 2 μl of 10 mM-Tris/HCl, 1 mM-EDTA (pH 7.4), 3 μl of potassium acetate and magnesium acetate to optimize their concentration (90–110 and 1.6–2.0 mM final concns. respectively) and 5 μl of rabbit reticulocyte lysate N90 were incubated for 1 h at 30 °C, and luciferase activity as chemiluminescent (CL) count was measured in a home-built luminometer (Campbell, 1988) for 10 s at room temperature in 229 μl of 20 mM-Tris/acetate/0.3 mM-dithiothreitol/0.2 mM-EDTA/1 mg of BSA/ml/12 mM-magnesium acetate/1.5 mM-ATP, pH 7.75. The reaction was started by addition of luciferin to 0.2 mM final concentration (1 ng of extracted luciferase yields 2.1×10^5 and 2.9×10^5 CL counts/10 s in the presence of rabbit reticulocyte lysate and buffer respectively). The amount of protein synthesized was measured by including 15 μCi of [^{35}S]methionine/10 μl of translation cocktail. Proteins were separated on SDS/9 % (w/v) polyacrylamide gels under reducing conditions (Laemmli, 1970) followed by fluorography and exposure to preflashed X-ray film. The luciferase bands were excised from the gel, radioactivity was measured in a liquid-scintillation counter and the amount of protein was estimated taking into account the concentration of methionine (28 μM) in the lysate.

Phosphorylation of proteins

The proteins were synthesized in 100–250 μl of rabbit reticulocyte mixture, precipitated in 64 % saturated ammonium sulphate, resuspended in 100 μl of 50 mM-Tris/Mes/1 mM-EDTA/0.3 mM-dithiothreitol (pH 7.8) for normal, and for protein with kemptide *N*-terminus (KNt) and kemptide at *C*-terminus (KCt) and pH 7.2 for RRFS, and subjected to gel filtration on a column (0.7 cm × 20 cm) packed with Sephacryl S100 and equilibrated in the corresponding buffer. Active fractions were pooled and concentrated by ultrafiltration. Protein was measured by the method of Lowry *et al.* (1951). BSA (fraction V) was used as standard.

The phosphorylation was carried out in a mixture containing 20 mM-Mes, 60 mM-sodium glycerol 2-phosphate, 30 mM-NaF, 10 mM-magnesium acetate, 1 mM-EDTA, 1 mg of BSA/ml, 1 μg each of leupeptin and pepstatin/ml and 125 μM-ATP, pH 6.8. Active fractions from the gel filtration (0.8–1.2 mg of protein/ml, of which approximately 0.1 % was luciferase) were added together with 0.5 μl of purified catalytic subunit of protein kinase A or catalytic subunit diluent (0.5 M-potassium phosphate/0.1 % Tween-20, pH 6.8) per 40 μl of mixture [the catalytic subunit can transfer 7 mmol of ^{32}P/min per μl using 20 μM-malantide as a substrate, as in Murray *et al.* (1990)]. The incubations were carried out at 30 °C for 10–20 min. Kemptide was also phosphorylated in the presence of rabbit reticulocyte that was gel-filtered under the same conditions as the variants in the presence of [γ-^{32}P]ATP (Livesey & Martin, 1988). The phosphorylated proteins were stored on ice until ready to assay.

Dephosphorylation of the luciferase

When the phosphorylated proteins were to be treated with alkaline phosphatase, the phosphorylation buffer contained no sodium glycerol 2-phosphate nor NaF. For the dephosphorylation reaction 0.7 unit of alkaline phosphatase/μl and 0.01 mM-protein kinase inhibitor (Cheng *et al.*, 1985) were added to the phosphorylation mixture.

Effect of pH on activity and colour of the light emitted by the variants

Chemiluminescence from the enzymes was measured by diluting them 40-fold into an assay mix with pH ranging from 6 to 9 containing mixtures of 50 mM-Mes and 50 mM-Tris to give the desired pH, 0.3 mM-dithiothreitol, 0.2 mM-EDTA, 1 mg of BSA/ml, 12 mM-magnesium acetate, 0.2 mM-luciferin and 1.5 mM-ATP. Colour was assessed using a dual-wavelength luminometer fitted with narrow-band pass-interference filters, with a maximal transmission at 603 nm (red) and 545 nm (green) of 30.2 and 35.3 % respectively (Campbell *et al.*, 1985). The light produced by the luciferase reactions was measured simultaneously at the two wavelengths and the ratio of activity at 603 nm to activity at 545 nm was calculated. The ratio was corrected for the transmission of the filters, but not for the spectral sensitivity of the photomultiplier tubes, which at 603 nm was approximately 10 % of its value at 545 nm.

RESULTS

Characterization of PCR products

The PCR was used to amplify cDNA coding for wild-type firefly luciferase and for variants containing putative protein kinase A-recognition sites at position 217–220 (referred to as RRFS), kemptide at *N*-terminus (referred to as KNt) or kemptide at *C*-terminus (referred to as KCt).

The PCR products were characterized using three criteria: size on agarose-gel electrophoresis, formation of ^{32}P-labelled mRNA of the correct size on glyoxal/agarose-gel electrophoresis and translation *in vitro* of the mRNA to form light-emitting protein. This protein was compared with wild-type synthetic luciferase for molecular mass, specific activity, pH profile and colour and with firefly tails luciferase when appropriate. The PCR generated a single DNA band apparently of the correct predicted size for all the recombinant DNA, i.e. for wild-type and RRFS the predicted size is 1682 bp, for KCt the predicted size is 1703 bp, and for KNt a major band is present at the predicted size 1703 bp with a minor band at 380 bp (Fig. 1). The yields were 1–3 μg of DNA/0.1 ml of reaction mixture. No bands were seen without addition of primers or when template DNA was omitted.

Transcription of the PCR products with T7 RNA polymerase generated a major band of ^{32}P-labelled capped mRNA of the correct length, i.e. 1650 bp. Small quantities of longer and shorter mRNA products were observed (Fig. 2). The latter could not generate light-emitting protein because removal of 12 amino acids at the *C*-terminus reduces the activity by 99 % (Sala-Newby *et al.*, 1990*b*). The yields of capped mRNA were 4–8 molecules of RNA per DNA molecule, the lower yields corresponding to DNA coding for KNt. No mRNA was detected in gels when the DNA transcribed lacked T7 promoter in spite of the detection of [^{32}P]UTP incorporation equivalent to 0.05 molecule of RNA per DNA molecule. mRNA generated light-emitting protein (Table 1) and a major ^{35}S-labelled protein band of the expected molecular mass (60 kDa) on SDS/PAGE (Fig. 3).

Characterization of the recombinant variants

The new proteins were characterized using three criteria: specific activity (i.e. CL counts/μg of RNA and CL counts/ng of protein), effect of pH 6–9 on their activity and colour of the light emitted as assessed by the ratio of chemiluminescence at 603 nm to 543 nm.

The CL counts/10 s per ng of protein indicated the effect the modifications had on the catalytic activity of the protein. Luciferase with kemptide at the *N*- or *C*-terminus had a specific activity similar to that of the wild-type and extracted luciferase. However, the specific activity of the RRFS variant was only 10–15 % of that of wild-type luciferase (Table 1). The specific chemiluminescent activity estimated per μg of RNA differed between the variants by a greater factor than the activity per μg

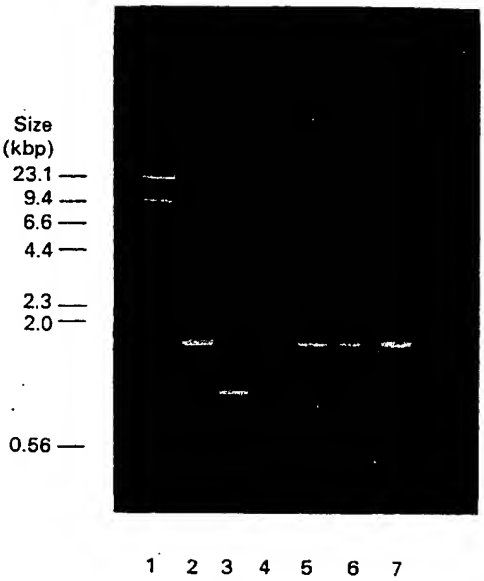

Fig. 1. Agarose-gel electrophoresis of cDNAs prepared by PCR

Wild-type firefly cDNA was amplified with oligonucleotides 105–100 (lane 2). The fragments of DNA that were used to prepare RRFS variant are shown in lanes 3 and 4. They correspond to PCR products obtained using oligonucleotide primers 107–100 (3′ end) and 101–108 (5′ end). The firefly RRFS cDNA is shown in lane 5; it was prepared by the amplification of DNA from lanes 3 and 4 in the presence of primers 105–100. cDNAs coding for variants with kemptide at *N*- and *C*-terminus are shown in lanes 6 and 7 respectively. Size markers were *Hin*dIII-digested λ DNA (lane 1).

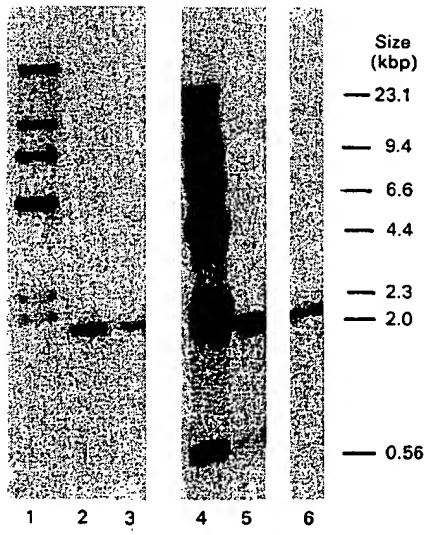

Fig. 2. Transcription products of the cDNAs

cDNAs produced by PCR were transcribed using T7 RNA polymerase and the ^{32}P-labelled mRNAs were separated by glyoxal/agarose-gel electrophoresis, dried and autoradiographed as described in the Materials and methods section. The size markers were ^{32}P-labelled *Hin*dIII-digested λ DNA (lanes 1 and 4) (Sambrook *et al.*, 1989). RNAs for the recombinant proteins are shown as follows: RRFS (lane 2), wild-type (lane 3), KNt (lane 5), KCt (lane 6).

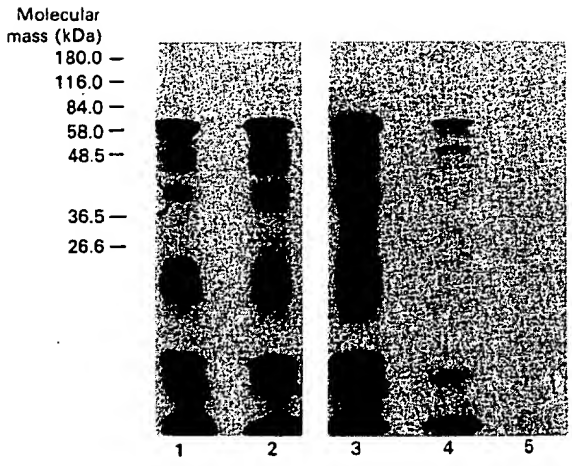

Fig. 3. Synthesis *in vitro* of recombinant proteins

mRNAs were translated using rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The proteins were separated by SDS/PAGE under reducing conditions. RRFS (lane 1), wild-type (lane 2), KCt (lane 3), KNt (lane 4) and the products in the absence of mRNA (lane 5) are shown. Prestained molecular-mass (Da) markers were: α_2-macroglobulin (180000), β-galactosidase (116000), fructose 6-phosphate kinase (84000), pyruvate kinase (58000), fumarase (48500), lactate dehydrogenase (36500) and triose phosphate isomerase (26600).

Table 1. Specific activity of the luciferases

The values in parentheses indicate the number of independent DNA amplifications. Results are expressed as means ± S.E.M. and when only two determinations were made the range is given.

Variant	CL counts/10 s per ng of protein	CL counts/10 s per μg of RNA	Protein/RNA (mol/mol)
Wild-type synthetic	$(2.6\pm0.5)\times10^5$ (3)	$(3.0\pm0.6)\times10^7$ (5)	1.04
RRFS	$(3.0\pm1.0)\times10^4$ (6)	$(3.9\pm1.3)\times10^5$ (6)	0.11
KNt	$(2.1–3.7)\times10^5$	$(4.2–4.7)\times10^6$	0.14
KCt	$(2.1–2.0)\times10^5$	$(3.7–8.9)\times10^6$	0.27
Extracted	2.1×10^5	–	–

of protein. All the variants with phosphorylation sites showed less activity per μg of RNA than the wild-type variant (Table 1). The number of molecules of protein produced per molecule of RNA in the translation assay, estimated from the two specific activities, confirmed that the normal synthetic wild-type enzyme yielded up to nine more copies of RNA than the other three (Table 1). The lower levels of translation shown could reflect differences in the secondary structure of the mRNA with the translation assay being optimized for the wild-type synthetic RNA. An additional effect due to a change in the codon usage cannot be ruled out. The RNA coding for KNt also contained an RNA band at approx. 500 bp (Fig. 2, lane 6) which would not translate into active protein as this would reduce the specific activity estimated per total RNA.

Effect of phosphorylation and dephosphorylation

Initial experiments using kemptide as a substrate for protein kinase A indicated that rabbit reticulocyte lysate inhibited phosphorylation of kemptide (results not shown). Gel filtration

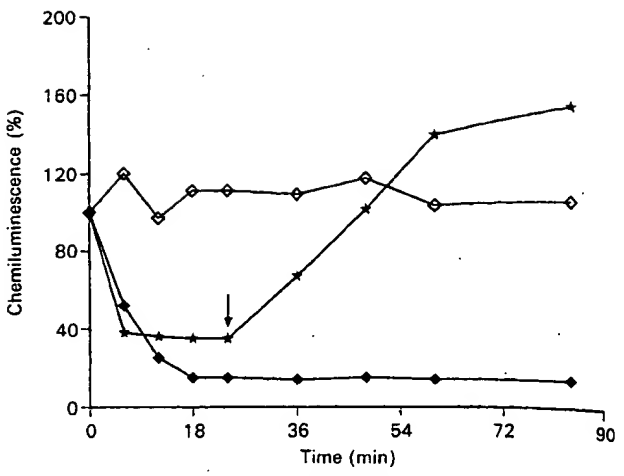

Fig. 4. Effect of phosphorylation–dephosphorylation on the activity of RRFS luciferase (V-217 → R)

Partially purified variant RRFS was incubated at 30 °C as described in the Materials and methods section in the presence of kinase diluent only (◇), protein kinase A catalytic subunit with (◆) and without (★) phosphate inhibitors. At 24 min alkaline phosphatase and protein kinase A inhibitor were added (↓). Samples were taken from the tubes at various times up to 90 min, diluted immediately 40-fold into luciferase assay mixture pH 7.2 and the chemiluminescence was measured for 10 s. Activity at time 0 was measured before addition of kinase. Results are presented as percentage of activity at time 0. Each point is a mean of two and a representative experiment is shown. Experiments were carried out with protein produced from two separate PCRs.

removed the inhibitory activity and 1.7 ± 0.2 ($n = 3$) nmol of phosphate (1.3–1.4 mol when phosphatase inhibitors were omitted) was incorporated into kemptide after 20 min incubation per 40 μl of reaction mixture.

Incubation of the RRFS luciferase variant with protein kinase A catalytic subunit in the presence of ATP resulted in a decrease in its catalytic activity to 19 ± 4% ($n = 5$) within 20 min, and remained at this level for the duration of the experiment, i.e. 90 min (Fig. 4). When alkaline phosphatase was added to the phosphorylated RRFS luciferase, the chemiluminescent activity increased to control levels within 30 min. No effect of protein kinase A was observed on the activity of wild-type luciferase, nor on recombinant luciferases with kemptide at the *N*- or *C*-terminus, at any pH (Figs. 5*a* and 5*b*).

Attempts to demonstrate a change in pI between the various recombinant luciferases, using isoelectric focusing, were unsuccessful, because of artifactual bands generated from the focusing procedure. However, the major band for recombinant and extracted luciferase had the same pI (6.6).

Recombinant wild-type luciferase had a pH optimum of around 7.8, identical with that of the extracted luciferase (Fig. 6*a*). Addition of kemptide at the *N*- or *C*-terminus appeared to have no effect on the pH profile (Figs. 5*b* and 6*a*). Similarly these three recombinant proteins had similar colour shifts to the red at acidic pH (Fig. 6*b*). In contrast the RRFS mutant luciferase showed both an altered pH profile with optimum activity at pH 7.2 (Figs. 5*a* and 6*a*) and a shift in colour to the green at acid pH (Fig. 6*b*). The inhibitory effect of phosphorylation on RRFS activity was most marked at its optimum pH (Fig. 5*a*). The ratio of light emission at 603 nm/543 nm measured at pH 7.5 changed from 0.16 to 0.32 after phosphorylation, indicating that the light became redder. Since the detection system used for activity measurements was less sensitive to red light, this red shift may

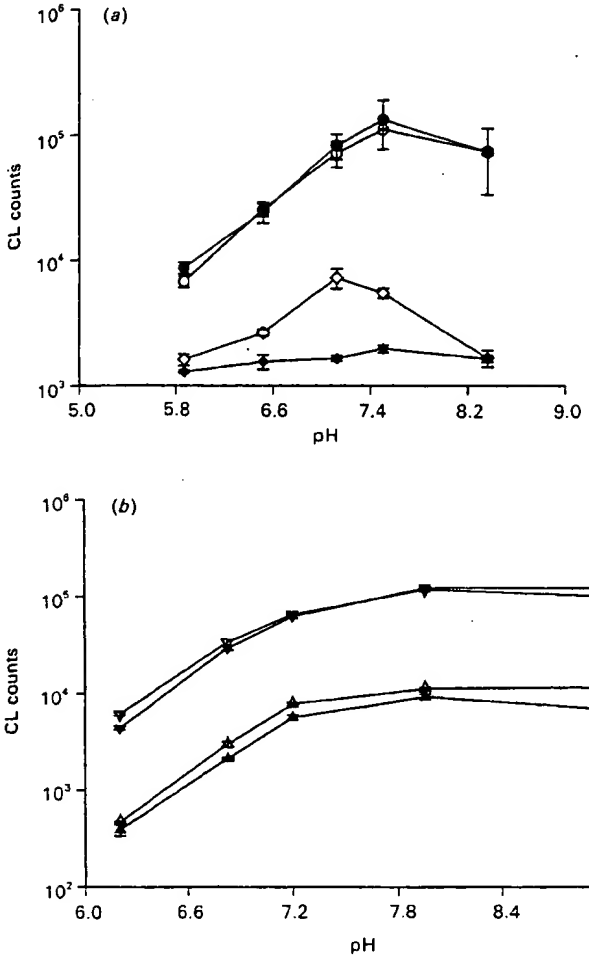

Fig. 5. Effect of pH and protein kinase A on the activity of recombinant luciferases

The variants were incubated for 15 min in the presence of protein kinase A (closed symbols) or kinase diluent only (open symbols) as described in the Materials and methods section. The resulting enzyme activity was then measured in triplicate (mean ± S.E.M.) at various pH values. (*a*) Wild-type (●, ○) and RRFS (◆, ◇). (*b*) KNt (▲, △) and KCt (▼, ▽).

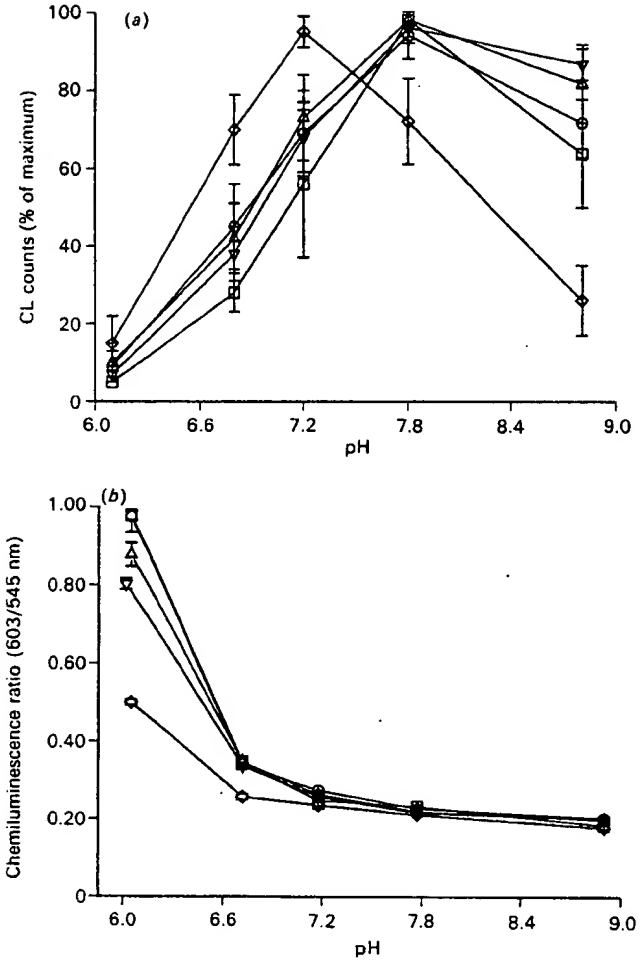

Fig. 6. Effect of pH on the activity and colour of the light emitted by luciferase variants

(*a*) pH optimum curve. The results are expressed as % of maximal activity (mean ± S.E.M.) from three to five experiments, each in triplicate. ○, Wild-type recombinant; □, extracted luciferase; ◇, RRFS; △, KNt; ▽, KCt. (*b*) pH effect on the colour of the light produced. The ratio of chemiluminescent counts at 603 nm and 545 nm was measured in triplicate at each pH (mean ± S.E.M.). ○, Wild-type recombinant; □, extracted luciferase; ◇, RRFS; △, KNt; ▽, KCt. The luciferase from firefly tails was purified as described by Sala-Newby *et al.* (1990*b*).

partly explain the decrease in activity for the phosphorylated enzyme.

DISCUSSION

The results presented demonstrate that DNA amplification coupled to transcription–translation *in vitro* allowed the generation and characterization of firefly luciferase variants containing phosphorylation sites. Only one of the variants (RRFS) showed a decrease in its activity when incubated with the catalytic subunit of protein kinase A in the presence of ATP, and the effect was reversed by addition of alkaline phosphatase (Figs. 4, 5*a* and 5*b*). The enzyme activity per unit of protein of the wild-type variant and the luciferases with kemptide at the *N*- or *C*-terminus were indistinguishable from that of the extracted luciferase (Table 1). The activity expressed per unit of RNA was more variable and lower for all the variants with phosphorylation sites than for the wild-type. The pH–activity profile for KNt, KCt and wild-type were very similar, but RRFS had a lower pH optimum (Fig. 6*a*). The colour of the light emitted was assessed by measuring the ratio of activities at 603 nm and 545 nm (Fig. 6*b*). At alkaline pH no significant differences were detected but as the pH was decreased the variant RRFS had a significantly lower ratio, indicating that the light emitted was greener than for all the others. As chemiluminometers contain photomultipliers which are more sensitive to green than red light this colour change cannot explain the decrease in specific activity measured. The activity was measured under saturating concentrations of ATP and luciferin, suggesting that the $V_{max.}$ was decreased.

Several beetle luciferases have now been cloned: *Photinus pyralis*, *Pyrophorus plagiophthalamus* and *Luciola cruciata* (de Wet *et al.*, 1987; Wood *et al.*, 1989*a*,*b*; Tatsumi *et al.*, 1989). Spectral changes are known to occur in the light emitted by firefly luciferase in response to changes in pH and temperature, and in the presence of heavy metals (Seliger & McElroy, 1964). Work on four click-beetle luciferases that show 94–99% sequence homology demonstrated that a small number of amino acid

substitutions were responsible for the different colours displayed by the luciferases. The spectral shift between luciferases yellow–green and yellow belong to the amino acid set, R-223, L-238 → E, V with the effect probably being due to R-223 → E (Wood *et al.*, 1989*a*,*b*). Since all the beetle luciferases use the same luciferin, the colour of the light emitted in the reaction must depend on the environment around the emitter (i.e. oxyluciferin). The oxyluciferin can exist as a monoanion (ketonic form) or dianion (enolic form) at acid and basic pH respectively. The presence of an arginine in position 223 of the click-beetle yellow–green luciferase seemed to be responsible for a shift to the green in the light it emitted. The change V-217 → R-217 that generated RRFS in *Photinus* luciferase introduced a basic amino acid in that area of the protein and also resulted in a shift to the green of the light emitted, suggesting that a positive charge there stabilized the oxyluciferin dianionic form, the green emitter.

The phosphorylation of the RRFS variant by the catalytic subunit of protein kinase A decreased its activity, and dephosphorylation reversed the effect. The decrease in activity was accompanied by a spectral shift to the red that can account for part of the lower activity measured. The other two variants, KNt and KCt, showed no detectable differences from the wild-type luciferase in any aspect. The luciferase with kemptide at the *C*-terminus was expected to show properties different from the normal luciferase in view of the fact that the removal of 12 amino acids at the *C*-terminus nearly abolishes activity (Sala-Newby *et al.*, 1990*b*). The removal of three amino acids (results not shown) and the addition of the seven amino acids from kemptide at the *C*-terminus did not affect the catalytic properties. This could be important when using firefly luciferase or its variant in eukaryotic cells because the last three amino acids of the *C*-terminus contain a peroxisomal targetting signal (Keller *et al.*, 1987; Gould *et al.*, 1987).

The RRFS variant provides, for the first time, an indicator potentially useful for measuring protein phosphorylation in intact cells, and has also highlighted a domain within the enzyme that results in changes in colour in response to a change in charge. Recognition peptides for other kinases could thus be engineered in this region of the protein, thereby establishing a universal strategy for measuring any protein kinase and visualizing it in living cells (Hooper *et al.*, 1990).

We thank Mr. Andrew Trimby for technical assistance, Dr. Rose Ann Padua for the synthesis of the oligonucleotides and both her and Dr. K. Murray for helpful discussions. This work was funded by the Medical Research Council and the Agriculture and Food Research Council.

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Received 25 April 1991/11 June 1991; accepted 18 June 1991